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PRINCIPAL INVESTIGATOR: Scott D. Kuduk, Ph.D.
Samuel Danishefsky, Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Institute for Cancer Research
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The development of efficient routes for the preparation of complex oligosaccharide or carbohydrate conjugates has been our immediate purpose. A more global goal would be that of recruiting the immune system to respond to malignant lesions. In our continuing chemical studies, we have undertaken to develop synthetic methodology of general applicability for the preparation of carbohydrates, in the form of glycolipids and glycopeptides, which mimic components of the accessible cell surface of tumor cells. The report herein focuses on successful syntheses of mucin related *O*-linked glycopeptides, in particular the TF and 2,3-STF antigens. TF is observed on a variety of epithelial tumors and 2,3-STF is observed on a large number of breast tumors.

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Scott Kudrik 6/1/99

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Progress Report **Scott D. Kuduk**

Synthesis and Clustering of the TF Dissacharide and 2,3-STF Trisaccharide

INTRODUCTION

The development of efficient routes for the preparation of complex oligosaccharide or carbohydrate conjugates has been our goal for some time. This goal would be that of recruiting the immune system to respond to malignant lesions. In our continuing chemical studies, we have undertaken to develop synthetic methodology of general applicability for the preparation of carbohydrates, in the form of glycolipids and glycopeptides, which mimic components of the accessible cell surface of tumor cells. The report herein focuses on mucin related *O*-linked glycopeptides, in particular the TF and 2,3-STF antigens. TF is observed on a variety of epithelial tumors and 2,3-STF is observed on a large number of breast tumors.

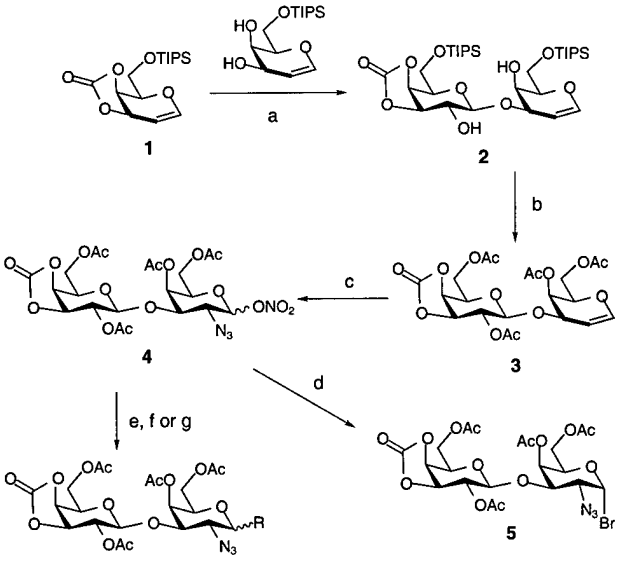
Mucins comprise a family of large glycoproteins expressed on cells of epithelial tissues that carry large glycodomains in clustered modes. Mucin amino acid sequences possess a very high percentage of serine and threonine residues, often found in contiguous arrays ranging in number from two to five. These amino acids which are glycosylated with the Thomsen-Friedenreich disaccharide (TF) antigen are quite common in carcinoma malignancies, particularly of the colon and prostate. Simple carbohydrate antigens have been synthesized and their immunogenicity in conjugate vaccines confirmed. For example, antibody titers against STn have been reported to correlate with improved prognosis in breast cancer patients. Comparable studies with more complex carbohydrates have rarely been described, thus the clear interest in large clustered forms of the TF and 2,3-STF antigens.

The first goal was the development of synthetic methodology to gain access to the clustered antigen motifs. We would go on from the chemistry phase to evaluate the immunogenicity of the clustered glycopeptide fragments in mice. The longer range goal would be the development and evaluation of antitumor glycopeptide-based vaccines. This report is concerned with work on the synthesis of TF and 2,3-STF.

Synthesis and Clustering of the TF Dissacharide

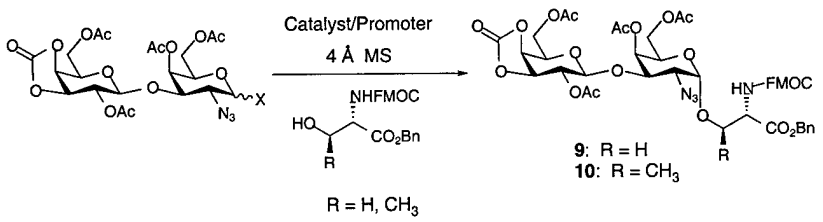
The first antigen investigated was the TF dissacharide. We then turned to the preparation of the TF disaccharide using the logic of glycal assembly based upon our experience with 1,2-anhydro sugars derived from protected galactal. The epoxide derived from treatment of **1** with DMDO in CH_2Cl_2, served as an excellent β-galactosyl donor with 6-TIPS galactal to afford disaccharide **2** in 80% yield. At this stage the protecting groups were exchanged from tri-isopropyl to acetate to afford **3**. The glycal underwent azidonitration efficiently, but with no selectivity, to furnish **4** as a 1:1 mixture of anomers in 67% yield. Azidonitration with the TIPS group present at the 6 and 6' positions gave a substantially lower yield (~30%) with significant byproduct formation. The azidonitrate was converted directly into the labile glycosyl bromide **5** with LiBr in CH_3CN or reduced by PhSH and DIEA in 91% yield. Treatment of **6** with DAST in THF afforded glycosyl fluoride **7** in 94% yield as a 1:1 mixture of α and β isomers, and trichloroacetimidate formation with Cl_3CCN and K_2CO_3 proceeded smoothly to give **8** as a separable 3:1 mixture of α and β isomers in 92% yield.

At this stage, the use of **5**,**7** and **8** in glycosylation reactions was investigated. Results are shown in Table 1. Without going into great detail here, the yields of the glycosylation were very good, but poor α/β selectivity was observed. The only positive feature of this study was that the α and β glycosylation products could be separated by careful chromatography.



6: R = OH
7: R = F
8: R = $OCNHCCl_3$

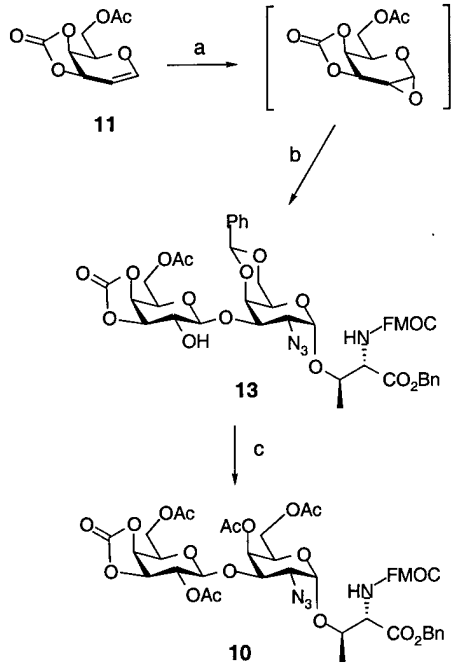
Reagents: (a) i) DMDO, CH_2Cl_2, 0°C; ii) $ZnCl_2$, THF, -78°C to rt overnight, 80%; b) i) TFAF-HOAc, THF, ii) Ac_2O, DMAP, TEA, 87%; c) NaN_3, CAN, CH_3CN, -15°C, 1 hr, 67%; d) LiBr, CH_3CN, 88%; e) PhSH, DIEA, CH_3CN, 0°C, 1hr, 85%; f) K_2CO_3, Cl_3CCN, CH_2Cl_2, rt, 17h, 92%; g) DAST, THF, -40°C, 1 hr, 94%

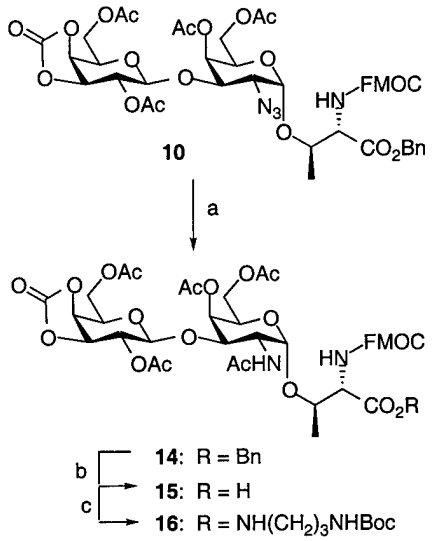


Donor X	Catalyst/Solvent/Temp	R = H α:β (yield)	R = CH_3 α:β (yield)
5 (Br)	AgOTf, CH_2Cl_2, -78°C to rt	-	1.2:1 (63%)
5 (Br)	$AgClO_4$, CH_2Cl_2, -78°C to rt	-	1.1:1 (73%)
7 (α-TCA)	TMSOTf (0.5 eq.), -30°C, CH_2Cl_2	1:1 (80%)	2:1 (80%)
7 (β-TCA)	TMSOTf (0.5 eq.), -30°C, CH_2Cl_2	1:1.3 (77%)	1.2:1 (72%)
7 (β-TCA)	TMSOTf (0.5 eq.), -50°C, CH_2Cl_2	-	1:1 (84%)
7 (β-TCA)	TMSOTf (0.5 eq.), -30°C, Et_2O:CH_2Cl_2 (12:1)	-	1:3.4 (75%)
8 (β/α-F)	Cp_2ZrCl_2, $AgClO_4$, CH_2Cl_2, -78°C to rt	-	1:1 (50%)

Bromide: Donor **5** (1.1 eq.) was added over 30 min to acceptor, catalyst (1.5 eq.) and 4 Å molecular sieves at -78°C and allowed to warm to rt overnight. TCA: Donor **7**, acceptor (1.5 eq.), and 4 Å molecular sieves were added in the indicated solvent and TMSOTf was added at the noted temperatures for 1 hr. Fluoride: Donor **8** (1.1 eq.) was added over 30 min to acceptor, catalysts (1.5 eq. of each) and 4 Å molecular sieves at -78°C and allowed to warm to rt overnight. All yields and ratios are for isolated products.

Plagued by this poor selectivity, a new method using a 'cassette' was developed. The idea was to use a glycosylated amino acid with the required α-*O*-linkage solidly in place as the



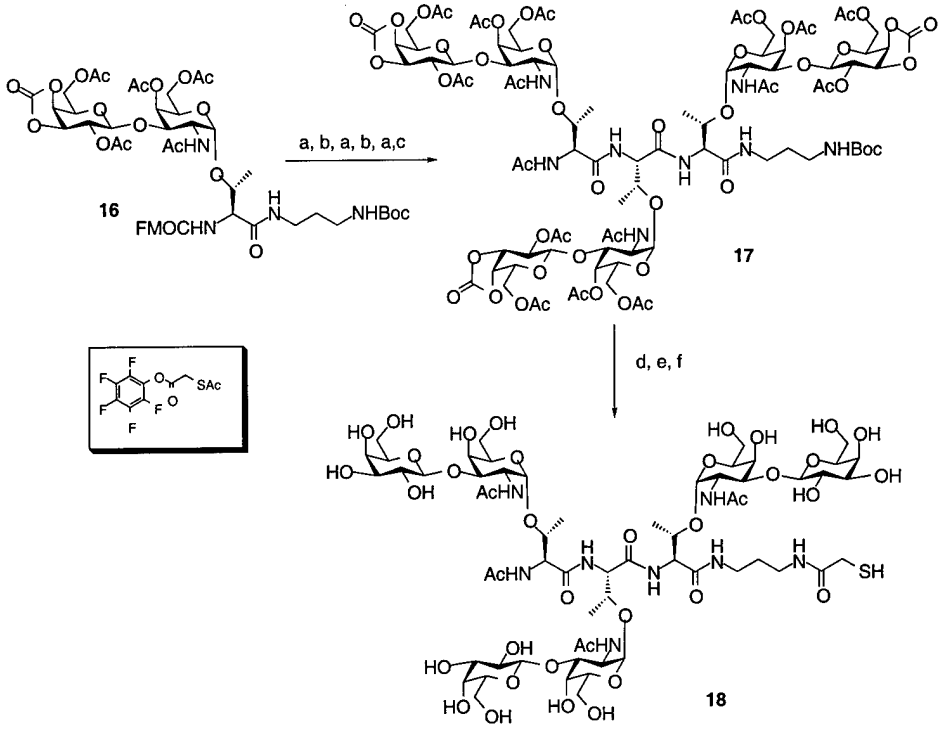


Reagents: (a) $CH_3C(O)SH$, 19 hr, 87%; (b) Pd/C, H_2, 2 hr, quant; (c) HOAt, HATU, Collidine, DMF,

glycosyl acceptor. Again, we turned to our glycal assembly methodology to simplify the construction. Thus, the epoxide derived from glycal **11** proved to be a powerful donor in reaction with **12** to afford β-linked disaccharide **13** in 97% yield. The disaccharide was readily converted to **10** in high overall yield.

Reductive acetylation of **10** was carried out with thiolacetic acid in 87% yield to afford protected TF antigen **14**. This step was followed by hydrogenolysis, leading to protected TF-acid **15** in quantitative fashion. At this point, we decided to attach the protected diamine linker first, and then to proceed on to the clustering. We did so because diminished yields were encountered for attachment of the linker at a late stage in the synthesis. This coupling to **15** initially failed when mediated by IIDQ, but proceeded in 84% yield with HOAT/HATU and collidine in DMF.

Glycopeptide **16** was then carried through the sequence of deprotections and coupling with **15** using the HOAt-HATU coupling method was required as IIDQ was extremely sluggish for this system. In addition, deprotection of the FMOC was facilitated using KF in DMF with catalytic amount of 18-Crown-6 as morpholine in DMF showed a proclivity to attack the 3,4-carbonate. After removal of the Boc group of **17** with TFA, coupling with SAMA-OPfp followed by NaOMe/MeOH deprotection afforded **18** in 60% yield for the three steps.

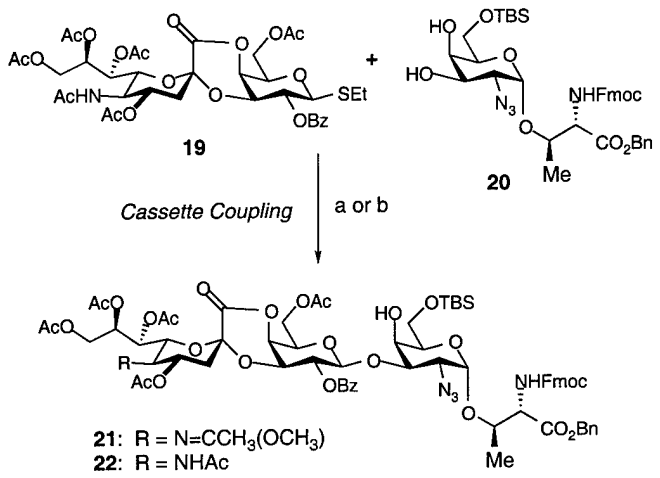


Reagents: (a) KF, DMF, 48 hr, 72-82%; (b) 47, HOAt, HATu, Collidine, DMF,75-84%; (c) Ac_2O, CH_2Cl_2; (d) TFA, CH_2Cl_2; (e) SAMA-OPfp, DIEA, CH_2Cl_2; (f) NaOMe, MeOH (degassed), rt, 60%.

Having completed the synthesis of **18**, the material was conjugated to a lipophilic Pam Cys immunostimulant and to KLH (keyhole limpet hemocyanin) protein immunostimulant. Both proved to give robust immune responses and the a vaccine (18-KLH plus Q-21) is now in a small clinical trial involving 30 patients. Results will be reported in due course.

Synthesis of the 2,3-STF Antigen

The synthesis of the 2,3-STF trisaccharide antigen proved to be much more complex than the corresponding TF disaccharide. Three unique factors had to be used. First, a cassette (**19**) similar to that used for TF, was required for the synthesis to completed. Second, it also required a specialized acceptor (**20**) in the form of a thioethyl glycoside as the epoxide used above for TF was unreactive in this system(not shown). And third, a special promoter (NIS/TfOH) was required as the benchmark promoter MeOTf led to unwanted methylation and low yields (shown below). We are currently in the process of clustering this more complex trisaccharide for clinical study.



Reagents: (a) MeOTf (10 eq.), CH_2Cl_2, 4Å mol sieves, 12-25% (**36**); (b) NIS, TfOH, CH_2Cl_2, 4Å mol sieves, 10 min, 62% (**37**).

Future goals

1- Large scale synthesis of the TF cluster **18** for larger patient trials
2- Clustering and immunological evaluation of the 2,3-STF cluster.
3- Synthesis of other antigenic structures.

Progress Report

Scott D. Kuduk

Synthesis and Clustering of the TF Dissaccharide and 2,3-STF Trisaccharide

Key accomplishments:

1-Synthesis of TF via conventional glycosylation strategy.
2-Development of a cassette approach to the synthesis of TF.
3-First ever reported clustering and immuno-evaluation of clustered TF.
4-Synthesis of 2,3-STF via a novel cassette approach.

Publications:

"Synthetic and Immunological Studies on Clustered Modes of Mucin Related Tn and TF *O*-linked Antigens.", **S.D. Kuduk**, J.B. Schwarz, X.T. Chen, D. Sames, P.W. Glunz, G. Ragupathi, P.O. Livingston and S.J. Danishefsky. *J. Am. Chem. Soc.* **1998**, *120*, 12474-12485.

"A Broadly Applicable Method for the Efficient Synthesis of α-*O*-Linked Glycopeptides and Clustered Glycopeptides", J.B. Schwarz, **S.D. Kuduk**, X.T. Chen, D. Sames, P.W. Glunz, and S.J. Danishefsky, *J. Am. Chem. Soc.* **1999**, *121*, 2662-2673.

"Probing Cell-Surface Architecture Through Synthesis: An NMR-Determined Structural Motif for Tumor-Associated Mucins", D. H. Live, L.J. Williams, **S.D. Kuduk**, J.B. Schwarz, P.W. Glunz, X.T. Chen, D. Sames, R.A. Kumar, and S.J. Danishefsky. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3489-3493.

PATENT: Please see following 'Title Page'

Synthetic and Immunological Studies on Clustered Modes of Mucin-Related Tn and TF O-Linked Antigens: The Preparation of a Glycopeptide-Based Vaccine for Clinical Trials against Prostate Cancer

Scott D. Kuduk, Jacob B. Schwarz, Xiao-Tao Chen, Peter W. Glunz, Dalibor Sames, Govindaswami Ragupathi, Philip O. Livingston, and Samuel J. Danishefsky

Contribution from the Laboratory for Bioorganic Chemistry and Laboratory for Tumor Vaccinology, Sloan-Kettering Institute For Cancer Research, 1275 York Avenue, New York, New York 10021, and Department of Chemistry, Columbia University, New York, New York 10027

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Synthetic and Immunological Studies on Clustered Modes of Mucin-Related Tn and TF O-Linked Antigens: The Preparation of a Glycopeptide-Based Vaccine for Clinical Trials against Prostate Cancer†

Scott D. Kuduk,‡ Jacob B. Schwarz,‡ Xiao-Tao Chen,§ Peter W. Glunz,‡ Dalibor Sames,‡,§ Govindaswami Ragupathi,⊥ Philip O. Livingston,⊥ and Samuel J. Danishefsky*,‡,§

Contribution from the Laboratory for Bioorganic Chemistry and Laboratory for Tumor Vaccinology, Sloan-Kettering Institute For Cancer Research, 1275 York Avenue, New York, New York 10021, and Department of Chemistry, Columbia University, New York, New York 10027

Received July 16, 1998

Abstract: The syntheses of two tumor-associated carbohydrate antigens, Tn and TF, have been achieved using glycal assembly and cassette methodologies. These synthetic antigens were subsequently clustered (c) and immunoconjugated to a carrier protein (KLH or BSA) or a synthetic lipopeptide (pam) for immunological study. Three Tn conjugates were used to vaccinate groups of mice, and all preparations proved to be immunogenic. The Tn(c) covalently linked to KLH (**27**−KLH) plus the adjuvant QS-21 was the optimal vaccine, inducing high median IgM and IgG titers against Tn(c) by ELISA. These antibodies were strongly reactive with the Tn(c) positive human colon cancer cell line LS-C but not the Tn(c) negative colon cancer cell line LS-B by FACS. The antibodies' reactivities with natural antigens were inhibited with synthetic Tn(c) but not with structurally unrelated compounds. On the basis of these results, vaccines containing **27**−KLH and **30**−pam plus QS-21 are being tested in patients with prostate cancer.

Introduction

The development of efficient routes for the preparation of complex oligosaccharide or carbohydrate conjugates has been our goal for some time.[1] Synthetic investigations in this area can help to provide a detailed knowledge of the structural and chemical behavior of carbohydrates and their conjugates. Furthermore, issues related to biological function of glycoconjugates can be evaluated, provided suitable quantities of informative probe structures can be constructed.

With time, we became interested in using chemistry to examine an exciting possibility. The goal would be that of recruiting the immune system to respond to malignant lesions. We were particularly drawn to the concept of inducing "active immunity" by synthetic vaccines.[2] Peptidic molecules, whose immunobiology has been studied extensively, have been used in the search for synthetic antitumor and other vaccines.[3] Such investigations had been greatly aided by preparative powers of peptide chemistry, and particularly by solid phase synthesis of peptides and recombinant technology. However, immune responses to nonpeptidic substances, such as carbohydrates, although highly abundant on surfaces of viruses, bacteria, and tumor tissues, remain poorly understood partly due to a lack of experimentation on telling probe structures. This situation reflects, in many instances, the complexity in the synthetic methodology required to reach adequate quantities of informative goal systems. In our continuing chemical studies, we have undertaken the development of synthetic methodology of general applicability for the preparation of carbohydrates, in the form of glycolipids and glycopeptides, which mimic components of the accessible cell surface of tumor cells. The disclosure herein focuses on mucin-related O-linked glycopeptides, in particular the Tn and TF antigens.

Mucins, which comprise a family of large glycoproteins expressed on cells of epithelial tissues, carry large glycodomains in clustered modes.[4] Mucin amino acid sequences possess a very high percentage of serine and threonine residues, often found in contiguous arrays ranging in number from two to five. In most cases, the details of the occupancy of such blocks of serine and threonine subunits is not known in detail.[5] Despite a large variety of mucin glycostructures, the modality wherein the first residue, an *N*-acetylgalactosamine moiety, is linked to

† Abbreviations: BSA, bovine serum albumin; DAST, diethylaminosulfurtrifluoride; DIEA, diisopropylethylamine; ELISA, enzyme-linked immunosorbent assay; FMOC, fluorenylmethoxycarbonyl; GalNAc, *N*-acetylgalactosamine; IIDQ, 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihryoquinoline; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; KLH, keyhole limpet hemacyanin; pam, palmitoylcysteine; MBS, *m*-maleimimidobenzoyl-*N*-hydroxysuccinimide ester; NHS, *N*-hydroxysuccinimide; SAMA−OPfp, *S*-acetylthioglycolic pentafluorophenyl ester; TFA, trifluoroacetic acid; Tn(c), Tn cluster.

‡ Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute For Cancer Research.

§ Department of Chemistry, Columbia University.

⊥ Laboratory for Tumor Vaccinology, Sloan-Kettering Institute For Cancer Research.

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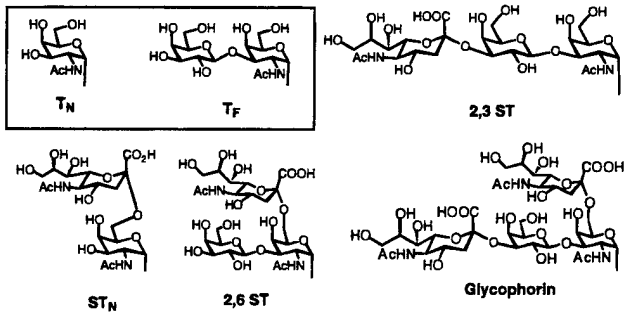

Figure 1.

a serine or threonine residue *via* an α-linkage appears to be broadly conserved (Figure 1). Interestingly, the significant changes of the glycopatterns during the malignant transformation generally result in shorter carbohydrate chains.[6] The Tn antigen represents the simplest member of the family. This antigen, as well as the related Thomsen–Friedenreich disaccharide (TF) antigen, is quite common in carcinoma malignancies, particularly of the colon and prostate.[7] Simple carbohydrate antigens have been synthesized, and their immunogenicity in conjugate vaccines has been confirmed.[8] For example, antibody titers against STn have been reported to correlate with improved prognosis in breast cancer patients.[9] Comparable studies with more complex carbohydrates have rarely been described, thus the clear interest in large clustered forms of antigens.

Given this context, we set for ourselves several goals. The first was the development of synthetic methodology to gain access to the clustered antigen motifs. We would go on from the chemistry phase to evaluate the immunogenicity of the clustered glycopeptide fragments in mice. The longer range goal would be the development and evaluation of antitumor glycopeptide-based vaccines.[10] In this paper we report the realization of these goals up to the point of the clinical trials which have just begun.

Synthetic Analysis

We have previously demonstrated that even bulky glycosyl amino acids can be efficiently incorporated into peptide backbones.[11] In light of these results, we opted to construct the glycosyl amino acids representing the Tn and TF epitopes. The amino acid domains would be protected in a fashion which anticipates the requirements of peptide assembly to form the clustered epitopes as shown in Figure 2. These constructs,

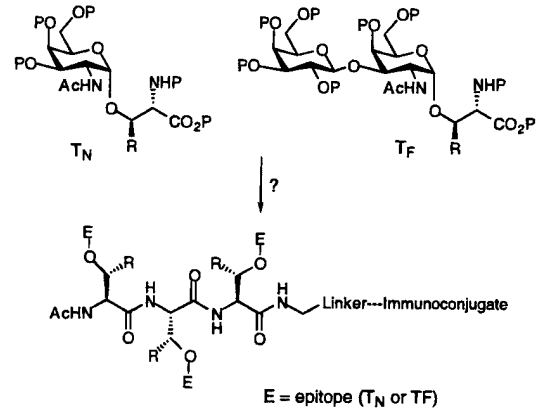

Figure 2.

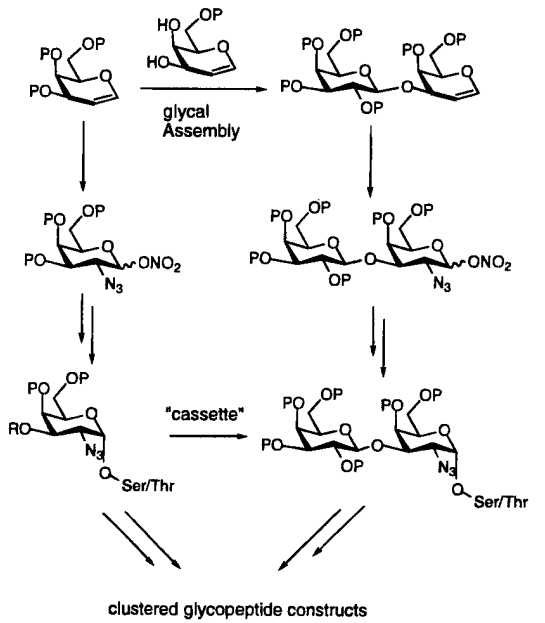

Figure 3.

suitably conjugated to immunogenic stimulants such as bacterial lipopeptides or carrier proteins, would then be evaluated in biological studies for antibody production, and eventual clinical application.

Synthetic Planning

For the synthesis of the Tn and TF antigens, we turned to our basic glycal assembly[1] logic. The key starting point would be the appropriately protected galactal, which could be used to produce both mono- and disaccharides. Azidonitration[12] of these glycals according to Lemieux might be used to introduce the 2-azido group and to produce the functionality at the anomeric carbon required for the fashioning of the prerequisite donor. Subsequent glycosylation of such a construct with the appropriately protected serine or threonine would pave the way for reaching our conjugates. An alternative and potentially more general method would involve building a shorter Tn construct as a "cassette", which would have the α-O-linked amino acid prebuilt into the GalNAc. This cassette might serve as a general acceptor to be inserted in the late stages of synthesis of virtually any O-linked glycopeptide goal structures. Such an approach, captured in Figure 3, might sidestep the serious difficulty of designing *ad hoc* methods for achieving high anomeric selectiv-

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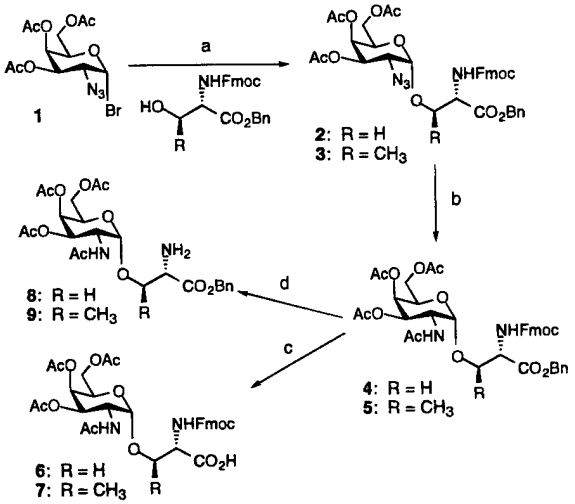
Scheme 1



Reagents: (a) $AgClO_4$, CH_2Cl_2, rt, 4:1 α:β; (b) AcSH, rt; (c) Pd/C, H_2, MeOH, H_2O, 75%; (d) 20% morpholine in DMF, 5 min.

ity in glycosylations of the side chain hydroxyls of serine and threonine for each new construct we hope to build.[13]

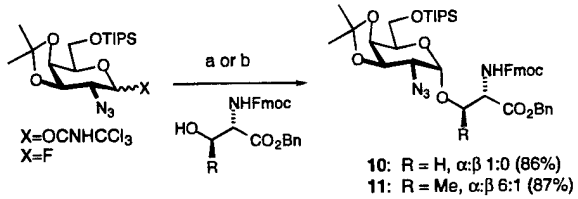
Results and Discussion

The Tn antigen had previously been prepared by a number of methods.[14] We chose a method to prepare the peracetylated form of the antigen, which is compatible with both solution and solid phase peptide chemistry. Thus, glycosylation of known bromide **1**[15] with *N*-Fmoc-serine or threonine benzyl ester afforded **2** or **3** as a separable 4:1 α/β mixture of anomers in 60% yield (Scheme 1). Reductive acetylation using neat thiolacetic acid produced fully protected Tn antigens **4** and **5**. Careful hydrogenolysis afforded the peracetylated acids **6** and **7** in 75% yield, while Fmoc removal gave **8** and **9**, which were in principle available for glycopeptide assembly.

The mediocre yield of glycosylation and the serious inconvenience of the need for anomer separation prompted a search for an alternative method. As matters turned out, another program in our laboratory, moving forward, had been directed to a total synthesis of the F1α antigen.[16] In screening for efficient serine or threonine glycosylation reactions, it was found that compounds **10** and **11** could be prepared as shown in Scheme 2. In the case of serine-derived acceptor the glycosylation ratio apparently gave only α-product **10**. In the synthesis of the threonine product **11**, a small amount of β-product was noted. In both cases the yields were far superior to those obtained from **1**. The idea then emerged to use **10** and **11** as general inserts (cassettes) to be installed toward the end of a complex synthesis. Thus, we need only solve the very difficult O-linkage problem once for a given "reducing end" and exploit that capability for building on the desired clustered system.

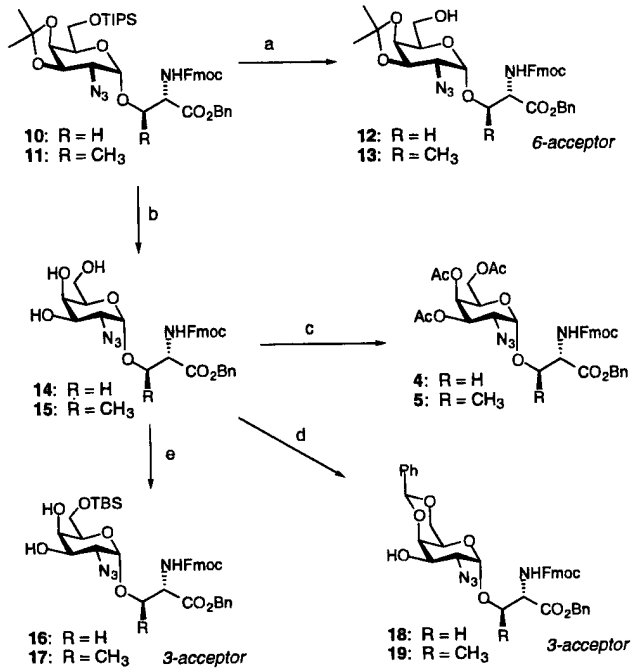
To implement this strategy, it would be necessary to fashion from the cassettes a variety of orthogonally protected modules for further use as glycosyl acceptors. In the case at hand, simple

Scheme 2



Reagents: (a) R = H, TMSOTf, THF, -78°C; (b) Cp_2ZrCl_2, AgOTf, CH_2Cl_2

Scheme 3



Reagents: (a) TBAF, AcOH, THF (94-100%); (b) I_2 / MeOH (63-81%); (c) Ac_2O, DMAP, TEA, CH_2Cl_2, (90%); (d) $PhCH(OMe)_2$, *p*-TsOH, CH_3NO_2 (73-94%); (e) TBSCl, Imidazole, DMF (64-85%).

TBAF-mediated desilylation of **10** or **11** affords position 6 acceptor **12** or **13**, respectively, in excellent yield (Scheme 3). Moreover, removal of both the TIPS and acetonide groups using I_2 in MeOH[17] gave rise to the versatile triol **14** or **15**, which could readily be converted to the peracetyl Tn precursors **4** and **5**. Alternatively the triols could be transformed, via resilylation with TBSCl, into diol 3 acceptor **16** or **17**,[18] or by benzylidene formation[19] to give specific position 3 acceptor **18** or **19**. We have used this methodology to prepare substantial quantities of the Tn antigen. In addition, we have used these intermediates for the efficient preparation of several O-linked tumor-associated antigens such as TF (vide infra) and sialosyl-Tn and sialosyl-T antigens.[20]

The amino acid sequence we chose for these clusters was to have three consecutive serines or threonines. Due to the lack of reliable information regarding which serine or threonine residues within a contiguous array constitutes an optimal epitope, we selected such trimers for the initial evaluation. The synthesis

(13) For reviews on the synthesis of α-O-linked serine or threonine glycopeptides, see: Kunz, H. *Pure Appl. Chem.* **1993**, *65*, 123−1332. Kunz, H. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 277.

(14) For examples of previous Tn syntheses, see: Toyokuni, T.; Hakomori, S.; Singhal, A. K. *Bioorg. Med. Chem.* **1994**, *11*, 1119 and references therein.

(15) (a) Paulsen, H.; Holck, J. P. *Carbohydr. Res.* **1982**, *109*, 89−107. (b) Paulsen, H.; Kolar, C. *Chem. Ber.* **1981**, *114*, 306.

(16) Chen, X. T.; Sames, D.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 7700−7709.

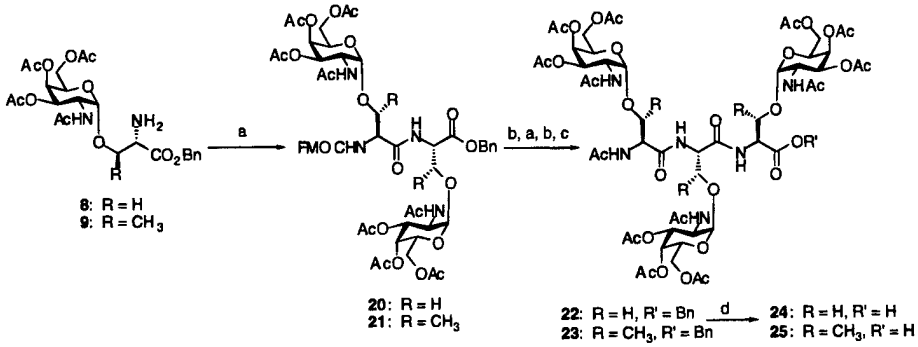
(17) Szarek, W. A.; Zamojski, A.; Tiwari, K. N.; Isoni, E. R. *Tetrahedron Lett.* **1986**, *27*, 3827.

(18) Nakahara, Y.; Nakahara, Y.; Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1997**, *38*, 7211−7214.

(19) Meinjohanns, E.; Meldal, M.; Schleyer, A.; Paulsen, H.; Bock, K. *J. Chem. Soc., Perkin. Trans.* **1996**, *1*, 985−993.

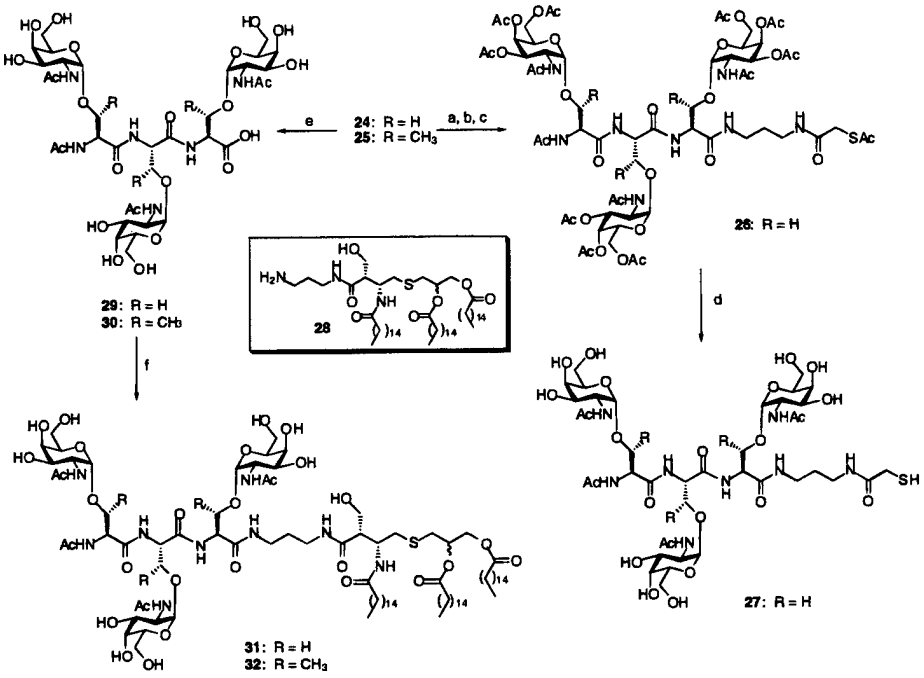
(20) Schwarz, J. B.; Kuduk, S. D.; Chen, X. T.; Sames, D.; Glunz, P. W.; Danishefsky, S. J. *J. Am. Chem. Soc.*, submitted.

Scheme 4



Reagents: (a) **6/7**, IIDQ, CH_2Cl_2, 85-97%; (b) 20% morpholine in DMF, 90-100%; (c) Ac_2O, CH_2Cl_2, 70-76%; (d) Pd/C, H_2, MeOH, H_2O, 85-95%.

Scheme 5



Reagents: (a) $H_2N(CH_2)_2NHBoc$, IIDQ, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) SAMA-(OPfp), DIEA, CH_2Cl_2, 81%; (d) NaOMe, MeOH (degassed), 85%; (e) NaOH, MeOH, 95%; (f) **29**, NHS, EDC, DMF, DIEA or HOAt, HATu, DMF, collidine, 40%.

of the clustered Tn glycopeptides began with IIDQ-mediated coupling between acid **6** or **7** and amine **8** or **9** to afford the corresponding dipeptides (see **20** and **21**) in 97% yield (Scheme 4). The Fmoc-carbamate was then deprotected with neat morpholine, and the resulting amine was resubjected to a second IIDQ coupling. This step was followed by deprotection and acetyl capping of the N-terminus to afford the tripeptides (see **22** and **23**). The glycosylated tripeptide thus obtained was subjected to hydrogenolysis to afford acids (see **24** and **25**), which could now be further modified and conjugated to either a synthetic lipopeptide as the immunological activator or to an immunogenic carrier protein.

As shown in Scheme 5, two pathways were followed for eventual conjugation. The first involved attachment of a suitable linker to conjugate with a carrier protein. The mercaptoacetamide unit has proven to be effective for this purpose.[21] Acid **24** was coupled with *tert*-butyl-*N*-(3-aminopropyl)carbamate *via* the agency of IIDQ. This step was followed by removal of the BOC cap with TFA. The resulting amine was then coupled with *S*-acetylthioglycolic acid pentafluorophenyl ester (SAMA-OPfp, NovaBiochem) in the presence of Hunig's base in 81% two-step yield. The resulting fully protected glycopeptide **26** was then subjected to methanolysis under carefully controlled conditions (pH ~9, degassed MeOH) to give **27** in 85% yield. The latter was now ready to be conjugated to the appropriate carrier protein.

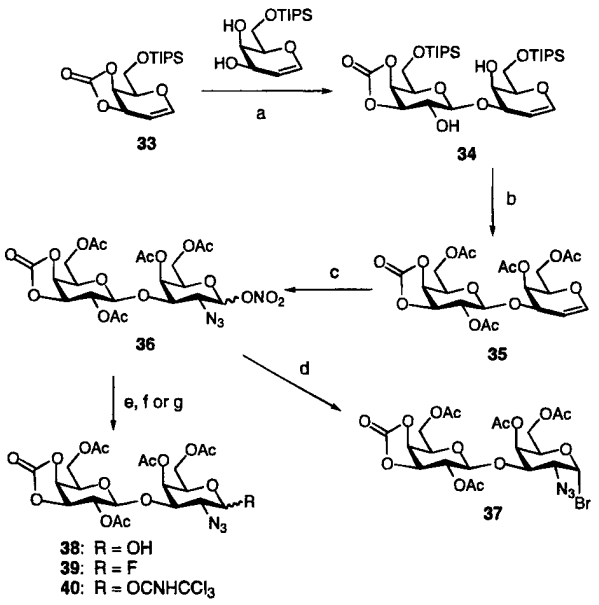
For the synthesis of a fully synthetic lipopeptide, we followed Toyokuni's method attaching tripalmitoyl-*S*-glycerylcysteinylserine (Pamcys).[22] Pamcys has proven to be a potent macrophage and B lymphocyte activator, and has been used for purposes similar to ours by Tokoyuni with one to three epitopes of serine Tn.[23] First, careful saponification of **24** or **25** with NaOMe/MeOH gave the fully deprotected glycopeptide **29** or **30** in 95% yield. Coupling with amine **28** using either the NHS or HOAt/HATU method[24] afforded glycolipid **31** or **32** in ca. 40% yield. Fortunately, the starting materials for this reaction can be recovered.

(21) Brugghe, H. R. *Int. J. Peptide Protein Res.* **1994**, *43*, 166.

(22) (a) Toyokuni, T.; Dean, B.; Cai, S.; Boivin, D.; Hakomori, S.; Singhal, A. K. *J. Am. Chem. Soc.* **1994**, *116*, 395. (b) Toyokuni, T.; Hakomori, S.; Singhal, A. K. *Bioorg. Med. Chem.* **1994**, *11*, 1119.

(23) Toyokuni, T.; Singhal, A. K. *Chem. Soc. Rev.* **1995**, 231–242.

Scheme 6



Reagents: (a) i) DMDO, CH_2Cl_2, 0°C; ii) $ZnCl_2$, THF, -78°C to rt overnight, 80%; b) i) TBAF-HOAc, THF, ii) Ac_2O, DMAP, TEA, 87%; c) NaN_3, CAN, CH_3CN, -15°C, 1 hr, 67%; d) LiBr, CH_3CN, 88%; e) PhSH, DIEA, CH_3CN, 0°C, 1hr, 85%; f) K_2CO_3, Cl_3CCN, CH_2Cl_2, rt, 17h, 92%; g) DAST, THF, -40°C, 1 hr, 94%

Synthesis of Clustered TF Antigen. We then turned to the preparation of the TF disaccharide using the logic of glycal assembly based upon our experience with 1,2-anhydro sugars derived from protected galactal. The epoxide derived from treatment of **33** with DMDO in CH_2Cl_2 served as an excellent β-galactosyl donor with 6-TIPS galactal to afford disaccharide **34** in 80% yield[25] (Scheme 6). Related approaches for the synthesis of the TF antigen disaccharide have been reported using glycosyl bromides and fluorides[26] or trichloroacetimidates as the galactosyl donors.[27] At this stage the protecting groups were exchanged from triisopropylsilyl to acetate to afford **35**. The glycal underwent azidonitration[12] efficiently, but with no selectivity, to furnish **36** as a 1:1 mixture of anomers in 67% yield. Azidonitration with the TIPS group present at the 6 and 6′ positions gave a substantially lower yield (~30%) with significant byproduct formation. The azidonitrate was converted directly into the labile glycosyl bromide **37** with LiBr in CH_3CN or reduced by PhSH and DIEA in 91% yield. Treatment of **38** with DAST[28] in THF afforded glycosyl fluoride **39** in 94% yield as a 1:1 mixture of α and β isomers, and trichloroacetimidate formation with Cl_3CCN and K_2CO_3 proceeded smoothly to give **40** as a separable 3:1 mixture of α and β isomers in 92% yield.[29]

At this stage, the use of **37**, **39**, and **40** in glycosylation reactions was investigated. Results are shown in Table 1. Several interesting observations should be noted. Glycosylation with *N*-Fmoc-threonine benzyl ester and bromide **37** afforded **42**, but gave essentially no selectivity with either AgOTf or $AgClO_4$, contrary to our previous observations for the 2,6-ST case.[11] Similarly, poor selectivities have been reported for TF glycosyl bromides and chlorides.[30] Likewise, with both α and β trichloroacetimidates **40**, no meaningful selectivity was observed under a variety of conditions, but yields were generally good to excellent.[31] The glycosyl fluoride **39** also gave no selectivity and a lower yield. In the past, threonine has in many cases been observed to give a much higher α-selectivity than serine. This was not the case for the systems described here. Also to be noted that the use of BF_3 as a promoter, as well as the use of THF as solvent, afforded no product. Another observation revealed a curious substrate effect. Thus, with ether as the solvent, in the case at hand a 3.4:1 β:α ratio was observed. By contrast in the case of the seemingly similar substrate **43** (Figure 4), α-product was favored 3:1 for this LeY-related system.[32] The only positive feature of this study was that the α and β glycosylation products could be separated by careful chromatography.[33]

Plagued by this poor selectivity, the cassette route was mobilized for implementation. The idea was to use a glycosylated amino acid with the required α-O-linkage solidly in place as the glycosyl acceptor. Again, we turned to our glycal assembly methodology to simplify the construction. Thus, the epoxide derived from glycal **44** proved to be a powerful donor in reaction with **19** to afford β-linked disaccharide **45** in 97% yield (Scheme 7). The disaccharide was readily converted to **42** in high overall yield. The use of such acceptors had been previously reported by Paulsen for the synthesis of the TF antigen, using glycosyl bromides[34] or trichloroacetimidates.[35] The Paulsen method is now complemented by the highly efficient approach directly from the glycal. It should be noted that conversion from 6-TIPS to a 6-acetate on the galactal carbonate was necessary for our glycosylation reaction to proceed.

Reductive acetylation of **42** was carried out with thiolacetic acid in 87% yield to afford protected TF antigen **46** (Scheme 8). This step was followed by hydrogenolysis, leading to protected TF acid **47** in quantitative fashion. At this point, we decided to attach the protected diamine linker first, and then to proceed on to the clustering.[36] We did so because diminished yields were encountered for attachment of the linker at a late stage in the synthesis. This coupling to **48** initially failed when mediated by IIDQ, but proceeded in 84% yield with HOAt/HATU and collidine in DMF.[37]

Glycopeptide **48** was then carried through the sequence of deprotections and coupling with **46** (Scheme 9) similar to that with Tn except using the HOAt–HATU coupling method[37] was required as IIDQ was extremely sluggish for this system. In

(24) (a) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397. (b) Carpino, L. A.; Ionescu, D.; El-Faham, A. *J. Org. Chem.* **1996**, *61*, 2460.

(25) Park, T. K.; Kim, I. J.; Hu, S.; Bilodeau, M. T.; Randolph, J. T.; Kwon, O.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1996**, *118*, 11488.

(26) (a) Kunz, H.; Birnbach, S.; Wernig, P. *Carbohydr. Res.* **1990**, *202*, 207–223. (b) Tsuda, T.; Nishimura, S. I. *Chem. Commun.* **1996**, 2779–2780. (c) Qui, D.; Gandhi, S. S.; Koganty, R. R. *Tetrahedron Lett.* **1996**, *37*, 595–598.

(27) Paulsen, H.; Peters, S.; Bielfeldt, T.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1995**, *268*, 17–34.

(28) (a) Rosenbrook, W., Jr.; Riley, D. A.; Larty, P. A. *Tetrahedron Lett.* **1985**, *26*, 3. (b) Posner, G. H. and Haines, S. R. *Tetrahedron Lett.* **1985**, *26*, 5.

(29) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Biochem.* **1994**, *50*, 84–123.

(30) (a) Kunz, H.; Birnbach, S.; Wernig, P. *Carbohydr. Res.* **1990**, *202*, 207–223. (b) Paulsen, H.; Peters, S.; Bielfeldt, T.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1995**, *258*, 17–34.

(31) Kinzy, W.; Schmidt, R. R. *Carbohydr. Res.* **1987**, *166*, 265–276.

(32) Glunz, P. W.; Hintermann, S.; Danishefsky, S. J. Unpublished results.

(33) High α-selectivities have been observed for amino acid glycosylation by Koganty through use of a 4,6-benzylidene acetal on the GalNAc as a "block donor", but the yields are slightly reduced: (a) Qui, D.; Gandhi, S. S.; Koganty, R. R. *Tetrahedron Lett.* **1996**, *37*, 595. (b) Yule, J. E.; Wong, T. C.; Gandhi, S. S.; Qui, D.; Riopel, M. A.; Koganty, R. R. *Tetrahedron Lett.* **1995**, *38*, 6839.

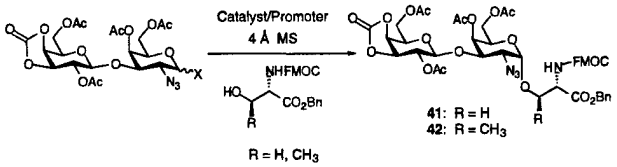
(34) Paulsen, H.; Holck, J. P. *Carbohydr. Res.* **1982**, *109*, 89–107.

(35) Meinjohanns, E.; Meldal, M.; Schleyer, A.; Paulsen, H.; Bock, K. *J. Chem. Soc., Perkin. Trans.* **1996**, *1*, 985–993.

(36) Mono- and diclusters of the TF antigen have been reported: Kunz, H.; Birnbach, S.; Wernig, P. *Carbohydr. Res.* **1990**, *202*, 207–223.

(37) (a) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397. (b) Carpino, L. A.; Ionescu, D.; El-Faham, A. *J. Org. Chem.* **1996**, *61*, 2460.

Table 1. [a]



donor X	catalyst/solvent/temp	R = H α:β (yield, %)	R = CH_3 α:β (yield, %)
37 (Br)	AgOTf, CH_2Cl_2, −78 °C to rt		1.2:1 (63)
37 (Br)	$AgClO_4$, CH_2Cl_2, −78 °C to rt		1.1:1 (73)
40 (α-TCA)	TMSOTf (0.5 equiv), CH_2Cl_2, −30 °C	1:1 (80)	2:1 (80)
40 (β-TCA)	TMSOTf (0.5 equiv), CH_2Cl_2, −30 °C	1:1.3 (77)	1.2:1 (72)
40 (β-TCA)	TMSOTf (0.5 equiv), CH_2Cl_2, −50 °C		1:1 (84)
40 (β-TCA)	TMSOTf (0.5 equiv), Et_2O/CH_2Cl_2 (12:1), −30 °C		1:3.4 (75)
39 (β/α-F)	Cp_2ZrCl_2, $AgClO_4$, CH_2Cl_2, −78 °C to rt		1:1 (50)

[a] Bromide: Donor **37** (1.1 equiv) was added over 30 min to acceptor, catalyst (1.5 equiv), and 4 Å molecular sieves at −78 °C and allowed to warm to rt overnight. TCA: Donor **40**, acceptor (1.5 equiv), and 4 Å molecular sieves were added in the indicated solvent, and TMSOTf was added at the noted temperatures for 1 h. Fluoride: Donor **37** (1.1 equiv) was added over 30 min to acceptor, catalysts (1.5 equiv of each), and 4 Å molecular sieves at −78 °C and allowed to warm to rt overnight. All yields and ratios are for isolated products.

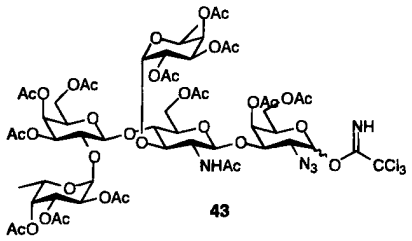
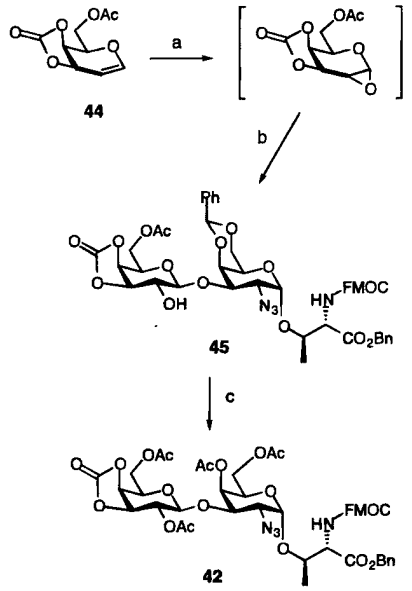


Figure 4.

Scheme 7

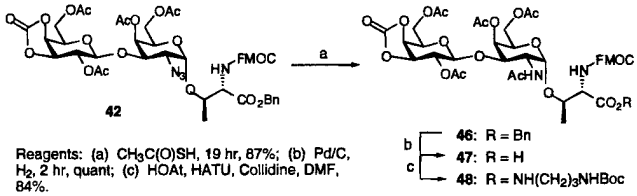


Reagents: (a) DMDO, CH_2Cl_2, 0°C, (b) **19**, $ZnCl_2$, THF, -78°C to rt, 97%; (c) i) 80% AcOH, 70°C, 3 hr, ii) Ac_2O, DMAP, TEA, CH_2Cl_2, 93%.

addition, deprotection of the FMOC was facilitated using KF in DMF with catalytic amount of 18-crown-6 as morpholine in DMF showed a proclivity to attack the 3,4-carbonate.[38] After removal of the Boc group of **49** with TFA, coupling with SAMA−OPfp followed by NaOMe/MeOH deprotection afforded **50** in 60% yield for the three steps.

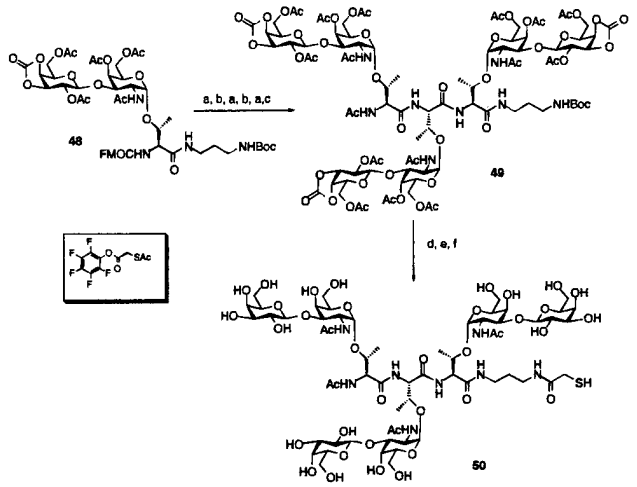
(38) Jiang, J.; Li, W.-R.; Joullie, M. *Synth. Commun.* **1994**, *24*, 187–195.

Scheme 8



Reagents: (a) $CH_3C(O)SH$, 19 hr, 87%; (b) Pd/C, H_2, 2 hr, quant; (c) HOAt, HATU, Collidine, DMF, 84%.

Scheme 9



Reagents: (a) KF, DMF, 48 hr, 72-82%; (b) 47, HOAt, HATu, Collidine, DMF,75-84%; (c) Ac_2O, CH_2Cl_2; (d) TFA, CH_2Cl_2; (e) SAMA-OPfp, DIEA, CH_2Cl_2; (f) NaOMe, MeOH (degassed), rt, 60%.

Discussion of Immunological Result

The initial experiments were to evaluate the antibody response to vaccination of mice with either Tn(c) lipopeptide **30** or more conventional Tn(c)−KLH or −BSA conjugates. The preparation of these conjugates started with the previously described **27**, which was covalently linked with carrier proteins, keyhole limpet hemocyanin (KLH), or bovine serum albumin (BSA), using MBS (*m*-maleimimidobenzoyl-*N*-hydroxysuccinamide ester), a heterobifunctional reagent which cross-links thiol groups with amino groups (Scheme 10). For KLH, about 317 clusters per protein were introduced, while BSA showed only 7 clusters per protein.

These conjugates plus the adjuvant QS-21 or Tn(c)−pam (**30**) in intralipid and **30** in intralipid plus QS-21 were used to

Scheme 10

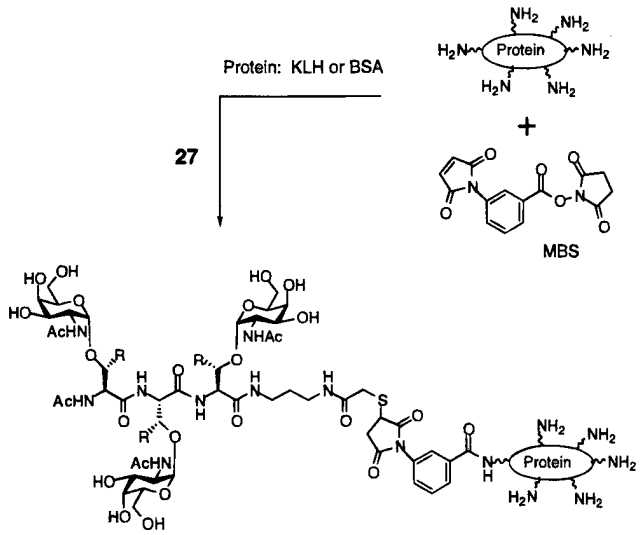


Table 2. ELISA Antibody Titers against Tn(c)[a]

	prevaccination		after third vaccination	
vaccine	IgM	IgG	IgM	IgG
30	0	0	1350	150
30 + QS-21	0	0	1350	50
27−KLH	0	0	**12150**	**450**
27−BSA	0	0	1350	150

[a] All titers are medians for groups of five mice.

vaccinate groups of five mice. All of these constructs proved to be immunogenic. There were no detectable anti-Tn(c) IgM or IgG antibodies present prior to vaccination. The median IgM and IgG ELISA titers against Tn(c)−pam in sera from the five groups of mice immunized with **27**- or **30**-conjugated vaccines at three weeks are shown in Table 2. Sera of mice immunized with 10 μg of **30** in conjunction with 10 μg of QS-21 failed to show strong reaction. In contrast to the case with **30**, construct **27** conjugated with KLH or BSA induced high IgM and moderate IgG titers. The highest titers were elicited by the KLH vaccine. IgM antibody titers remained higher than IgG titers at most time points, including the aftermath of two booster immunizations. In general, titers were no higher after the booster immunizations than after the initial immunizations.

Immunogenic protein carriers[39] have been among the most well studied approaches to the problem of increasing immunogenicity of carbohydrate antigens. Such protein conjugate vaccines have elicited high IgM and moderate IgG titer antibodies against the colon ganglioside GM2 in clinical trials.[40] The studies here with Tn(c) vaccines were patterned after previous studies with ganglioside vaccines, and the results were similar. Of the five carriers and many adjuvants tested, KLH was found to be the most effective carrier and QS-21, a homogeneous saponin fraction purified from the bark of *Quillaja saponaria* Molina, the most effective adjuvant. KLH conjugation plus the use of QS-21 as adjuvant was also the approach

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Table 3. Tn Cluster FACS Analysis: Serum Tested 11 Days Post Third Vaccination

group	IgG (tested 1/9/98) % positive cells	IgM (tested 1/12/98) % positive cells
30	46.99	39.98
30 + QS-21	12.00	46.41
27−KLH + QS-21	94.72	49.54
27−BSA + QS-21	92.14	51.89

found optimal previously for augmenting the immunogenicity of ganglioside GD3 and GM2.[41]

The cell surface reactivity of anti-Tn(c) antibodies was evaluated using Tn(c) positive LS-C colon cancer cells and Tn(c) negative LS-B colon cells. Measurements involved flow cytometry assays and complement dependent cytotoxicity (CDCX) assays. The median percent positive cells by flow cytometry with sera from mice **30** or **30** with QS-21-vaccinated mice was low. However, sera from mice vaccinated with **27**−KLH or **27**−BSA with QS-21 showed clear IgM reactivity with LS-C colon cancer cells by flow cytometry (Table 3). IgG reactivity was also seen by flow cytometry.

Important characteristics of the antibody response to immunization with **27**−KLH conjugate plus QS-21 included the pattern of antibody titers and the specificity of the antibodies. IgM antibody titers were significantly higher than IgG at most time points, despite repeated booster immunizations. There was evidence for neither an IgM to IgG class switch nor a secondary antibody response, all consistent with the T cell independent antibody responses characteristic of most carbohydrate antigens. Specificity analysis of the IgM antibody responses using inhibition assays demonstrated that the response was polyclonal with antibody subpopulations recognizing several different clustered epitopes. Tn(c) expressed on cells was readily recognized by the induced antibodies, resulting in complement activation and lysis of Tn(c) positive tumor cells. More detailed study on the immunology of these vaccines will be described elsewhere.

Summary

The synthesis and clustering of Tn and Tf antigens has been achieved. The application of glycal assembly and further development of the "cassette" methodology has laid the foundation for application to more complex O-linked glycopeptide synthesis. This report confirms the immunogenicity of such fully synthetic and characterized complex synthetic carbohydrate antigens for use in vaccines. It provides a basis for the synthesis and testing of other complex carbohydrate vaccines such as TF.[42] On the basis of these observations we have initiated clinical trials with the **27**−KLH with QS-21 vaccine in patients with Tn(c) positive cancers. Early indication suggests that the synthetic vaccines are well tolerated. Immunological and clinical data will be forthcoming in due course.

Experimental Section

Materials. A cysteine group was introduced to facilitate conjugation with protein carriers. QS-21[43] was obtained from Aquila Biopharmaceutical, Inc. (Worcester, MA). Keyhole limpet hemocyanin (KLH) was obtained from PerImmune Inc. (Rockville, MD). Bovine serum

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albumin (BSA) and sodium cyanoborohydride were obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibody IE3 (mAb IE3) was kindly provided by Dr. Singhal. Goat anti-mouse IgG and IgM conjugated with alkaline phosphatase and goat anti-mouse IgM fluorescence isothiocyanate (FITC) were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL). Female CB6F1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

General Procedure for Glycosylation of 1. A flame-dried flask was charged with silver perchlorate (930 mg, 2 equiv), 4.0 g of 4 Å molecular sieves, and *N*-Fmoc-L-threonine benzyl ester (1.21 g, 2.8 mmol, 1.2 equiv) in a drybox. A 30 mL sample of dry CH_2Cl_2 was added to the flask, and the mixture was stirred at rt for 10 min. Donor **1** (1.13 g, 2.87 mmol) in 16 mL of CH_2Cl_2 was added slowly over 30 min via syringe. The reaction was stirred under argon atmosphere at rt overnight. The mixture was then diluted with CH_2Cl_2 and washed twice with water. The solution was dried over Na_2SO_4 and evaporated, and the crude material (a 4:1 mixture of α/β isomers) was purified on a silica gel column (1−1.5−2−2.5% MeOH/CH_2Cl_2) to provide **3** (1.28 g, 60% yield).

Compound 2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.28−7.42 (m),), 5.94 (d, J = 8.0 Hz, 1H), 5.39 (d, J = 2.6 Hz, 1H), 5.21−5.27 (m), 4.86 (d, J = 3.4, 1H), 4.58−4.62 (m, 1H), 4.40 (d, J = 7.2 Hz, 2H), 4.24 (t, J = 7.1, 1H), 4.16 (dd, J = 3.0, 10.9 Hz, 1H), 3.50−4.08 (m), 3.58 (dd, J = 3.5, 11.2 Hz, 1H),), 2.14 (s, 3H), 1.96 (s, 3H). All data are in agreement with literature reports.[14]

Compound 3. Compound **15** (0.58 g, 0.94 mmol) was taken up in 5.0 mL of acetic anhydride, and then 1.0 mL of pyridine was added. The solution was stirred at ambient temperature for 1 h and then partitioned cautiously between 50 mL of EtOAc and 50 mL of 1 N HCl(aq). The phases were separated, and the organic phase was washed with saturated $NaHCO_3$ (2 × 50 mL) and brine (50 mL), dried (Na_2SO_4), and concentrated. Flash chromatography of the residue (2:1 hexanes/EtOAc) furnished 0.63 g (90%) of **3** as a colorless foam: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.6 Hz, 2H), 7.27−7.42 (m), 5.66 (d, J = 9.5 Hz, 1H), 5.44 (br s, 1H),), 5.25−5.31 (m),), 5.37 (d, J = 13.2, 1/2AB, 1H), 5.20 (d, J = 12.1 Hz, 1/2AB, 1H), 4.90 (d, J = 3.6 Hz, 1H), 4.40−4.51 (m), 4.31−4.38 (m), 4.18−4.28 (m), 3.70 (d, J = 6.5 Hz, 2H), 3.58 (dd, J = 3.7, 11.2 Hz, 1H), 2.16 (s, 3H), 2.04 (s, 3H), 1.34 (d, J = 6.3 Hz, 3H). All data are in agreement with literature reports.[14]

Compound 4. To a round-bottom flask were added α-glycoside **2** (1.25 g, 1.71 mmol), pyridine (2 mL), and thiolacetic acid (2 mL) at 0 °C subsequently. The reaction was stirred from 0 °C to room temperature overnight. After evaporation of the solvent by a flow of air, the residue was separated by chromatography to give desired product (1.13 g, 88%): $[\alpha]_D^{20}$ +61.0° (c 0.68, $CHCl_3$); IR (film) 3346, 3018, 2954, 1748, 1732, 1715, 1668, 1557, 1538 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.72 (d, J = 7.5 Hz, 2H), 7.56 (d, J = 7.5 Hz, 2H), 7.25−7.45 (m, 9H), 6.06 (d, J = 7.8 Hz, 1H), 5.74 (d, J = 8.8 Hz, 1H), 5.30 (d, J = 3.0 Hz, 1H), 5.16 (m, 2H), 5.03 (dd, J = 11.2, 3.0 Hz, 1H), 4.76 (br s, 1H), 4.59 (m, 1H), 4.49 (m, 1H), 4.40 (d, J = 6.8 Hz, 2H), 4.20 (t, J = 6.8 Hz, 1H), 3.92-4.15 (m, 5H), 2.12 (s, 3H), 1.96 (s, 3H), 1.92 (s, 3H), 1.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 170.7, 170.2, 170.1, 170.0, 168.8, 155.8, 143.6, 141.2, 134.7, 128.7, 128.3, 127.7, 127.0, 124.9, 120.0, 99.1, 69.8, 68.1, 67.6, 67.2, 61.9, 54.6, 47.6, 47.0, 23.1, 20.6, 20.5; HRMS (FAB) calcd for $C_{39}H_{43}N_2O_{13}$ [M + H]$^+$ 747.2765, found 747.2766.

Compound 5. Compound **3** (0.40 g, 0.54 mmol) was taken up in 5.0 mL of thiolacetic acid and the solution stirred for 20 h at ambient temperature. The mixture was concentrated with a N_2 flow and the residue purified by flash chromatography (1:1 hexanes/EtOAc) to give 0.31 g (76%) of **5** as a colorless oil: $[\alpha]_D^{23}$ + 49.6° (c 1.26, $CHCl_3$); ^{1}H NMR ($CDCl_3$, mixture of rotamers) (major rotamer) δ 7.76 (d, J = 7.4 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.33 (m, 9H), 5.78 (m, 1H), 5.60 (m, 1H), 5.36 (br s, 1H), 5.18 (d, J = 11.8 Hz, 1H), 5.06 (d, J = 11.8 Hz, 1H), 4.78 (d, J = 3.1 Hz, 1H), 4.52 (m, 1H), 4.44 (m, 3H), 4.21 (m, 3H), 4.05 (m, 3H), 2.15 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H), 1.29 (d, J = 6.3 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 171.09, 171.01, 170.53, 170.47, 156.71, 143.84, 141.45, 134.52, 129.08, 129.02, 128.73, 127.95, 127.27, 125.33, 125.21, 120.19, 100.03, 77.14, 68.56, 68.00, 67.50, 67.33, 62.36, 58.88, 47.62, 47.28, 23.37, 20.87, 20.77, 18.38; IR (neat) 3332, 2978, 1748, 1677 cm^{-1}; HRMS calcd for $C_{40}H_{44}N_2O_{13}Na$ 783.2741, found 783.2756.

General Procedure B for Deprotection of Benzyl Esters. To a solution of **5** (0.46 g, 0.60 mmol) in 150 mL of methanol and 10 mL of H_2O was added 0.14 g of 5% Pd/C. The system was evacuated and purged 5× with H_2 and then placed under 1 atm of H_2 for 90 min. The suspension was gravity filtered and concentrated. Flash chromatography of the residue (10 → 15 → 20% MeOH/CH_2Cl_2) yielded 0.37 g (92%) of **7** as a colorless crystalline solid.

Compound 7. $[\alpha]_D^{23}$ + 90.6° (c 1.55, $CHCl_3$); ^{1}H NMR ($CDCl_3$) mixture of rotamers; spectra available in Supporting Information, confirmed by variable temperature NMR; ^{13}C NMR ($CDCl_3$, mixture of rotamers) δ 173.78, 173.51, 172.42, 171.52, 171.28, 170.58, 170.27, 170.22, 170.00, 157.73, 156.46, 143.51, 143.47, 143.21, 143.13, 141.24, 141.19, 141.16, 127.77, 127.69, 127.66, 127.12, 127.04, 126.96, 125.10, 124.77, 124.53, 124.46, 119.92, 119.85, 99.48, 98.71, 77.71, 77.20, 75.73, 68.01, 67.53, 67.26, 67.22, 66.96, 66.90, 62.15, 61.96, 58.61, 48.37, 47.77, 47.16, 46.84, 22.82, 22.12, 20.75, 20.62, 20.58, 20.54, 20.51, 18.41, 18.12; IR (neat) 3344, 2938, 1748, 1726 cm^{-1}; HRMS calcd for $C_{33}H_{38}N_2O_{13}Na$ 693.2272, found 693.2298.

General Procedure A for Deprotection of FMOC-carbamates. Compound **5** (0.18 g, 0.24 mmol) was taken up in 3.0 mL of morpholine and the solution stirred at ambient temperature for 30 min. Excess morpholine was then removed by azeotroping with dry toluene under reduced pressure (3 × 4 mL). Flash chromatography of the residue (4 → 7.5 → 10% MeOH/CH_2Cl_2) provided 0.11 g (86%) of amine **9** as a coloroless foam.

Compound 8. To a round-bottom flask were charged **4** (330 mg, 0.44 mmol) and morpholine (3 mL) at 0 °C. The reaction mixture was stirred at 0 °C to rt for 3 h. After evaporation of the solvent by a flow of air, the residue was separated by chromatography (4 → 7.5 → 10% MeOH/CH_2Cl_2) to give the desired amine (230 mg, 99%): $[\alpha]_D^{20}$ +62.0° (c 1.46, $CHCl_3$); IR (film) 3310, 2960, 1751, 1734, 1654, 1542 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.31−7.35 (m, 5H), 5.86 (d, J = 9.8 Hz, 1H), 5.30 (d, J = 2.3 Hz, 1H), 5.15 (d, J = 11.5 Hz, 1H), 5.12 (d, J = 11.5 Hz, 1H), 5.05 (dd, J = 11.4, 3.0 Hz, 1H), 4.78 (d, J = 3.6 Hz, 1H), 4.53 (m, 1H), 4.01−4.12 (m, 3H), 3.90 (dd, J = 9.8, 3.6 Hz, 1H), 3.66−3.70 (m, 2H), 2.12 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H), 1.92 (s, 3H), 1.80 (m, 2H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 173.5, 170.8, 170.3, 170.2, 170.1, 135.2, 128.8, 128.7, 128.3, 98.8, 71.0, 68.4, 67.3, 61.9, 54.8, 47.7, 23.1, 20.7, 20.6; HRMS (FAB) calcd for $C_{24}H_{33}N_2O_{11}$ [M + H]$^+$ 525.2070, found 525.2090.

Compound 9: $[\alpha]_D^{23}$ +55.9° (c 1.16, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5H), 6.01 (d, J = 9.5 Hz, 1H), 5.34 (d, J = 2.5 Hz, 1Hz), 5.16 (d, J = 12.0 Hz, 1H), 5.05 (d, J = 12.1 Hz, 2H), 4.77 (d, J = 3.7 Hz, 1H), 4.50 (m, 1H), 4.21 (m, 1H), 4.05 (m, 3H), 3.42 (br s, 1H), 2.13 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.93 (s, 3H), 1.65 (br s, 2H), 1.33 (d, J = 6.4 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 174.16, 170.68, 170.19, 134.75, 128.66, 128.43, 99.75, 78.03, 68.53, 67.28, 67.11, 67.04, 62.01, 59.06, 47.50, 22.99, 20.61, 20.49, 18.09; IR (neat) 3385, 2976, 1745, 1666 cm^{-1}; HRMS calcd for $C_{25}H_{34}N_2O_{11}Na$ 561.2060, found 561.2069.

Compound 18. To a solution of **14** (0.24 g, 0.40 mmol) in 5.0 mL of nitromethane was added benzaldehyde dimethyl acetal (0.11 mL, 0.79 mmol), followed by *p*-toluenesulfonic acid monohydrate (4.0 mg, 0.02 mmol). The mixture was stirred for 1 h at ambient temperature at which time an additional 0.11 mL of $PhCH(OMe)_2$ and 4.0 mg of TsOH were added. Stirring was continued for 2 h, and the mixture was neutralized with 5 drops of Et_3N and concentrated. Flash chromatography of the residue (2:1 hexanes/EtOAc) afforded 0.26 g (94%) of **18** as a colorless foam: $[\alpha]_D^{23}$ +79.9° (c 1.35, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.58 (m, 2H), 7.46 (m, 2H), 7.31−7.42 (m, 7H), 5.89 (d, J = 8.0 Hz, 1H), 5.50 (s, 1H), 5.25 (d, J = 12.1 Hz, 1H), 5.20 (d, J = 12.1 Hz, 1H), 4.90 (d, J = 3.2 Hz, 1H), 4.57 (m, 1H), 4.44 (dd, J = 10.5, 7.1 Hz, 1H), 4.32 (dd, J = 10.4, 7.3 Hz, 1H), 4.21 (app t, J = 7.2 Hz, 1H), 4.12−4.18 (m, 3H0, 3.98 (m, 2H), 3.86 (d, J = 12.7 Hz, 1H), 3.56 (br s, 1H), 3.49 (dd, J = 10.6, 3.3 Hz, 1H), 2.38 (d, J = 10.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 169.67, 155.85, 143.72, 143.58, 141.25, 137.17, 134.99, 129.31, 128.62, 128.53, 128.27, 127.74, 127.09, 127.05, 126.11, 125.07, 124.95, 120.02, 101.19, 99.97, 75.18, 69.81, 68.92, 67.77, 67.26, 66.93, 63.21, 60.41,

54.58, 46.97; IR (neat) 3423, 2921, 2109, 1721 cm^{-1}; HRMS calcd for $C_{38}H_{36}N_4O_9Na$ 715.2380, found 715.2387.

Compound 19. **19** was prepared as described for compound **18** and obtained in 73% yield from **15** as a colorless solid: $[\alpha]_D^{23}$ +90.4° (c 1.17, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.48 (m, 2H), 7.31−7.39 (m, 7H), 5.79 (d, J = 9.3 Hz, 1H), 5.54 (s, 1H), 5.23 (s, 2H), 4.93 (d, J = 3.4 Hz, 1H), 4.47 (m, 3H), 4.34 (dd, J = 10.5, 7.6 Hz, 1H), 4.23 (m, 3H), 4.09 (ddd, J = 13.3, 10.4, 3.1 Hz, 1H), 4.02 (d, J = 12.1 Hz, 1H), 3.68 (s, 1H), 3.53 (dd, J = 10.5, 3.4 Hz, 1H), 2.53 (d, J = 10.6 Hz, 1H), 1.29 (d, J = 6.3 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 170.06, 156.74, 143.86, 143.63, 141.24, 141.21, 137.19, 134.95, 129.35, 128.61, 128.51, 128.29, 127.67, 127.07, 127.05, 126.15, 125.16, 125.10, 119.92, 101.16, 99.20, 76.20, 75.24, 69.02, 67.70, 67.38, 67.28, 63.18, 61.03, 58.71, 47.08, 18.63; IR (neat) 3427, 2921, 2110, 1725 cm^{-1}; HRMS calcd for $C_{39}H_{38}N_4O_9Na$ 729.2537, found 729.2561.

General Procedure C for Peptide Couplings Using IIDQ. To a solution of amine **9** (0.11 g, 0.20 mmol) in 3.0 mL of CH_2Cl_2 at 0 °C was added quickly a solution of acid **7** (0.15 g, 0.23 mmol) in 3.0 mL of CH_2Cl_2. Immediately following the addition, IIDQ (74 mg, 0.25 mmol) in 1.0 mL of CH_2Cl_2 was added quickly dropwise. The mixture was allowed to warm slowly to ambient temperature and stirred for 24 h. The solvent was evaporated with an N_2 flow, and flash chromatography of the residue (EtOAc) afforded 0.23 g (97%) of dipeptide **21** as colorless crystals.

Compound 25. Benzyl ester **23** (0.21 g, 0.15 mmol) was deprotected according to general procedure B. Filtration and concentration of the crude reaction mixture provided 0.19 g (96%) of pure free acid **25** as a colorless crystalline solid: $[\alpha]_D^{23}$ +103.5° (c 1.76, $CHCl_3$); ^1H NMR (CD_3OD) δ 8.40 (d, J = 9.0 Hz, 1H), 8.31 (d, J = 9.2 Hz, 1H), 5.40 (m, 3H), 5.15 (m, 4H), 4.99 (d, J = 3.8 Hz, 1H), 4.91 (J = 3.7 Hz, 1H), 4.78 (d, J = 2.1 Hz, 1H), 4.70 (m, 1H), 4.62 (m, 1H), 4.31−4.46 (m, 10H), 4.11 (m, 6H), 2.14 (s, 3H), 2.13 (s, 3H), 2.13 (s, 3H), 2.12 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H), 1.93 (s, 3H), 1.93 (s, 3H), 1.38 (d, J = 6.2 Hz, 3H), 1.32 (m, 6H); ^{13}C NMR (CD_3OD) δ 173.83, 173.51, 173.49, 173.33, 173.16, 172.54, 172.44, 172.09, 172.06, 171.94, 101.25, 100.83, 100.45, 79.62, 77.86, 77.50, 70.45, 70.13, 69.77, 68.92, 68.87, 68.31, 68.25, 63.36, 63.14, 58.22, 58.14, 58.01, 23.41, 23.13, 23.08, 22.50, 20.74, 20.70, 20.66, 20.62, 20.58, 19.54, 19.35, 19.21; IR (neat) 3324, 2981, 1748, 1658 cm^{-1}; HRMS calcd for $C_{56}H_{82}N_6O_{32}Na$ 1373.4871, found 1373.4856.

Compound 26. Boc-protected 1,3-diaminopropane (12.1 mg, 0.07 mmol) in 1 mL of CH_2Cl_2 (anhydrous) was added to a flask charged with glycopeptide **24** (70 mg, 0.05 mmol). While the solution was being stirred, IIDQ (21.2 mg, 0.07 mmol) in 0.8 mL of CH_2Cl_2 was added at 0 °C. After 5 min the reaction was removed from the bath and stirred for 48 h. Longer reaction times are required for this coupling. The reaction mixture was purified directly on a silica column (7% MeOH/CH_2Cl_2). The product was dissolved in 0.2 mL of CH_2Cl_2, and 0.6 mL of CF_3COOH was added. The reaction was complete in 30 min. The solvent was removed with a stream of nitrogen, and the material was dried *in vacuo*. The crude product was dissolved in a 1:1 mixture of tBuOH/AcOH and lyophilized. To a solution of the crude ammonium salt (27 mg, 0.026 mmol) was added pentafluorophenyl acetylmercaptoacetate (9.1 mg, 1.5 equiv) in 0.7 CH_2Cl_2 and $(iPr)_2$-NEt (7.3 μL, 0.04 mmol). The mixture was purified by a silica column (3−4−5−6−7% MeOH, CH_2Cl_2) to yield pure product **26** (27 mg, 70% yield): ^1H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, J = 8.3 Hz), 7.72 (d, J = 7.9 Hz), 7.39−7.59 (m), 7.22 (d, J = 9.5 Hz), 7.10 (d, J = 8.9 Hz), 6.70−6.82 (m), 5.32−5.40 (m), 5.08−5.17 (m), 5.03 (d, J = 2.9 1H), 4.99 (d, J = 3.0 Hz, 1H), 4.97 (d, J = 3.3 Hz, 1H), 4.51−4.70 (m), 4.13−3.91 (m), 3.65−3.72 (m), 3.60 (d, J = 15.0 Hz), 1.95−2.08 (14 acetates), 1.42 (d, J = 7.14), 1.39 (d, J = 6.6 Hz); MS (ES) [M + Na] 1503.6, calcd for $C_{56}H_{84}N_8O_3Na$ 1503.6.

Compound 27. The protected glycopeptide **26** was dissolved in anhydrous MeOH (deoxygenated with a stream of nitrogen/argon in order to prevent oxidative dimerization!). The pH was adjusted to 9−10 with a 25% solution of NaOMe in MeOH, and the mixture was stirred under argon overnight. Acidic Amberlyst resin was then added until the solution became neutral or mildly acidic (pH 5−6). The solvent was removed, and the crude was purified by RP chromatography (C18 silica gel, eluted with H_2O). The solution was lyophilized to yield glycopeptide **27** (85%) as a white solid. Further analysis was renounced to avoid dimerization of the thiol, and the material was stored under Ar at −78 °C: MS (ES) [M + Na] 1083.4, calcd for $C_{40}H_{68}N_8O_{23}SNa$ 1083.4.

Compound 29. To a stirred solution of peracetylated cluster **24**[14] (0.19 g, 0.14 mmol) in 10.0 mL of methanol was added sodium methoxide (25 wt % solution in MeOH) until the pH of the solution reached 9 (ca. 10 drops). Stirring was continued for 16 h, at which time Amberlyst-15 was added to lower the pH of the mixture to ca. 4. The solution was separated from the resin *via* pipet, the resin was rinsed with methanol (3 × 5 mL), and the combined extracts were concentrated. Purification of the residue on LiChroprep RP-18 using 1:1 MeOH/H_2O as eluant furnished 0.13 g (95%) of tripeptide **29** as a colorless crystalline solid. $[\alpha]_D^{23}$ + 160.7° (c 1.30, $CHCl_3$); ^1H NMR (CD_3OD) δ 8.32 (J = 8.5 Hz, 1H), 8.24 (d, J = 8.8 Hz, 1H), 7.53 (m, 2H), 7.38 (m, 2H), 4.98 (d, J = 3.2 Hz, 1H), 4.81 (d, J = 3.5 H, 1H), 4.72 (br s, 1H), 4.66 (br s, 1H), 4.48 (s, 1H), 4.16−4.29 (m, 6H), 3.92 (m, 6H), 3.79 (m, 3H), 3.71 (m, 6H), 2.09 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.31 (m, 6H), 1.22 (d, J = 6.3 Hz, 3H); ^{13}C NMR (CD_3OD) δ 174.33, 174.24, 173.79, 172.69, 171.85, 101.09, 100.79, 78.24, 77.66, 77.37, 72.87, 72.81, 70.81, 70.43, 70.31, 62.76, 62.66, 58.58, 58.31, 57.92, 51.68, 51.59, 51.47, 23.48, 23.43, 23.25, 22.49, 19.32, 19.07, 19.03; IR (neat) 3372, 2933, 1633 cm^{-1}; LRMS calcd for $C_{38}H_{64}N_6O_{23}Na$ 995.3920, found 995.3886.

Compound 31. **31** was prepared as described for **32** in 40% yield: MS (ES) [M + Na] 1988.5, calcd for $C_{95}H_{172}N_{10}O_{30}S$ 1988.5. Data are in agreement with literature description.[14]

Compound 32. To a slurry of tripeptide **30** (52 mg, 0.053 mmol) and amine acetate **28** (100 mg, 0.090 mmol) in 4.0 mL of dry DMF at ambient temperature were added HATU (81 mg, 0.21 mmol), HOAt (14.5 mg, 0.11 mmol), and then diisopropylethylamine (37 μL, 0.21 mmol). The mixture was stirred at ambient temperature for 16 h, at which time the DMF was removed *in vacuo*. The residue was rinsed with CH_2Cl_2 (3 × 0.5 mL). The remainder of the residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH/H_2O, 65:25:4) to afford 40 mg (38%) of the lipid conjugate as a colorless amorphous solid. ^1H NMR ($CDCl_3$/CD_3OD/D_2O, 65:25:4) δ 8.37 (d, J = 9.3 Hz, 1H), 8.33 (d, J = 8.8 Hz, 1H), 8.03 (d, J = 7.1 Hz, 3H), 7.92 (m, 1H), 7.72 (app t, J = 8.2 Hz, 1H), 7.66 (d, J = 8.8 Hz, 1H), 7.32 (d, J = 9.2 Hz, 1H), 5.19 (m, 1H), 4.87 (d, J = 3.6 Hz, 1H), 4.83 (d, J = 3.6 Hz, 1H), 4.80 (d, J = 3.7 Hz, 1H), 4.67 (m, 2H), 4.57 (m, 2H), 4.13 (m, 6H), 3.89 (br s, 7H), 3.74 (m, 1H), 3.19 (m, 4H), 3.04 (m, 1H), 2.88 (m, 1H), 2.77 (m, 1H), 2.31 (m, 6H), 2.11 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.61 (m, 9H), 1.27 (s, 90H), 1.19 (d, J = 6.1 Hz, 3H), 0.88 (t, J = 6.6 Hz, 9H); LRMS calcd for $C_{98}H_{178}N_{10}O_{30}S$ 2007.2, found 2007.2.

Compound 36. To a solution of protected galactal **35** (1.01 g, 2.01 mmol) in 11 mL of anhydrous CH_3CN at −20 °C was added a mixture of NaN_3 (392 mg, 6.03 mmol) and CAN (3.30 g, 6.03 mmol). The reaction mixture was vigirously stirred at −15 °C for 1 h. Then the reaction mixture was diluted with diethyl ether, and washed with cold water and brine subsequently. Finally, the solution was dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by chromatography on silica gel (50−75% EtOAc/hexanes). A mixture of α and β anomers **36** (2.17 g, 67% yield) was obtained. The ratio of α and β anomers was almost 1:1 based on ^1H NMR: IR (film) 3476, 2963, 2119, 1813, 1747, 1660, 1372, 1227, 1051 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) 6.25 (d, J = 4.2 Hz, 1H, H-1, α anomer), 5.40 (d, J = 8.6 Hz, 1H, H-1, β anomer); MS (EI) calcd 629.2, found 629.2 (M + Na). Copies of ^1H and ^{13}C NMR spectra are available in the Supporting Information.

Bromide 37. A solution of compound **36** (220 mg, 0.363 mmol) in 1.0 mL of dry acetonitrile was mixed with lithium bromide (140 mg, 1.6 mmol, 5 equiv) and stirred at rt for 3 h in the dark. The heterogeneous mixture was diluted with dichloromethane, the solution was washed twice with water and dried over magnesium sulfate, and the solvent was evaporated without heating. After rapid flash chromatography on predried silica gel (EtOAc), α-bromide **37** (200 mg, 88%) was isolated and stored under argon atmosphere at −80 °C to

prevent hydrolysis unitl use: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.16 (d, J = 3.6 Hz, 1H), 5.63 (d, J = 2.8 Hz, 1H), 5.19−5.15 (m, 2H), 4.90−4.81 (m, 2H), 4.41−3.96 (m, 18H); CI (NH_3) MS calcd for (M + H) $C_{21}H_{27}N_3BrO_{14}$ 629, found 629.

Compound 38. To a solution of a mixture of azidonitrates **36** (390 mg, 6.64 mmol) in 3 mL of anhydrous CH_3CN at 0 °C were slowly added Et(i-Pr)$_2$N (111 μL, 0.64 mmol) and PhSH (198 μL, 1.9 mmol) subsequently. The reaction mixture was stirred at 0 °C for 1 h, and then the solvent was blown off by an argon flow. The residue was purified by chromatography on silica gel (EtOAc) to give a 1:1 mixture of α and β anomers of **38** (318 mg, 85%) as a white foam: ^{1}H NMR (400 MHz, $CDCl_3$) 5.35 (d, J = 3.4 Hz, 1H, H-1 α anomer), 4.86 (d, J = 8.4 Hz, 1H, H-1, β anomer); MS (EI) calcd 562.2, found 562.2 (M + H); FAB HRMS calcd for (M + Na) $C_{21}H_{27}N_3O_{15}Na$ 584.1340, found 584.1319. Copies of ^{1}H and ^{13}C NMR spectra are available in the Supporting Information.

Fluoride 39. To a solution of **38** (53 mg, 0.081 mmol) in 1 mL of THF was added 20 mL (0.12 mmol, 1.5 equiv) of DAST at −40 °C, and the reaction was allowed to warm to rt over 1 h. The reaction was then quenched with 0.5 mL of MeOH and purified by chromatography on silica gel (50−80% EtOAc/hexane) to afford 25 mg (47%) of **39**α and 26 mg of **39**β (47%). These were used immediately to prevent degradation of the donor. **39**α: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.68 (dd, J = 55, 2.5 Hz, 1H), 5.59 (d, J = 6.4 Hz, 1H), 5.12−5.10 (m, 2H), 4.86 (dd, J = 8.5, 1.2 Hz, 1H), 4.79 (dd, J = 8.5 Hz, 1.2 Hz, 1H), 4.39−4.17 (m, 4H), 4.13 (br t, J = 5.7 Hz, 1H), 4.06 (dd, J = 10.5, 2.9 Hz, 1H), 3.96−3.87 (m, 2H), 2.20 (s, 3 H), 2.15 (s, 3H), 2.11 (s, 3H), 2.03 (s, 3H); ^{19}F NMR (421 MHz, $CDCl_3$) δ 14.2 (dd, J = 55, 26.5, 1F). **39**β: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (br t, 1H), 5.13−5.06 (m, 2H), 4.94 (d, J = 7.6 Hz, 1H), 4.87 (dd, J = 8.6, 1.6 Hz, 1H), 4.80 (dd, J = 8.6, 1.6 Hz, 1H), 4.40−4.20 (m, 4H), 4.10 (dt, J = 6.5, 1.4 Hz, 1H), 3.98 (dd, J = 11.7, 1.6 Hz, 1H), 3.86−3.79 (m, 2H), 3.56 (dd, J = 10.3, 3.6 Hz, 1H), 2.21 (s, 3H), 2.18 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H); ^{19}F NMR (421 MHz, $CDCl_3$) δ 23.5 (dd, J = 54.8, 14.2, 1F): CI (NH_3) MS calcd for (M + H) $C_{21}H_{26}FN_3O_{14}$ 564, found 564.

Trichloroacetimidate 40. To a solution of **38** (327 mg, 0.58 mmol) in 5 mL of CH_2Cl_2 at 0 °C were added K_2CO_3 (300 mg) and Cl_3CCN (0.58 mL, 5.8 mmol). The reaction mixture was stirred at 0 °C to room temperature overnight. The suspension was filtered through a pad of Celite and washed with CH_2Cl_2. The filtrate was evaporated, and the residue was purified by chromatography on silica gel (40−80% EtOAc/hexane) to give α-trichloroacetimidate **40**α (94 mg, 22%) and β-trichloroacetimidate **40**β (270 mg 72%) as white foams. **40**β: $[\alpha]_{20}^{D}$ +36.8° (c 0.28, $CHCl_3$); IR (film) 3318, 2962, 2116, 1750, 1679, 1370, 1224, 1071 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (br s, 1H), 5.58 (d, J = 8.6 Hz, 1H), 5.47 (d, J = 3.2 Hz, 1H), 5.10−5.05 (m, 2H), 4.85−4.80 (m, 2H), 4.37 (dd, J = 10.2, 6.7 Hz, 1H), 4.28 (dd, J = 10.0, 6.6 Hz, 1H), 3.99−3.89 (m, 7H), 3.62 (dd, J = 10.3, 3.3 Hz, 1H), 2.21 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0, 170.3, 170.2, 169.9, 168.6, 160.6, 152.9, 100.1, 96.4, 90.1, 79.5, 73.0, 72.6, 72.3, 68.3, 67.6, 67.5, 62.1, 61.6, 60.2, 20.8, 20.6, 20.5, 14.0. **40**α: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 (br s, 1H), 6.45 (br s, 1H), 5.61 (br s, 1H), 5.13 (br t, 2H), 4.87 (d, J = 8.5 Hz, 1H), 4.85 (d, J = 8.5 Hz, 1H), 4.37−4.08 (m, 16H), 3.86 (dd, J = 8.2, 4.1 Hz, 1H), 2.17 (s, 3H), 2.12 (s, 3H), 2.07 (s, 3H), 1.99 (s, 3H); FAB HRMS calcd for (M + Na) $C_{23}H_{27}N_4O_{15}C_{13}Na$ 727.0436, found 727.0446.

General Procedure for Glycosylation with Glycosyl Bromide. A flame-dried flask was charged with silver perchlorate (185 mg, 0.896 mmol), 300 mg of 4 Å molecular sieves, and *N*-Fmoc-L-threonine benzyl ester (283 mg, 0.672 mmol, 1.5 equiv) in a glovebag. Then, 2.5 mL of CH_2Cl_2 was added to the flask, and the mixture was stirred at rt for 10 min. Donor **37** (280 mg, 0.448 mmol) in 2.5 mL of CH_2Cl_2 was added slowly over 1 h. The reaction was stirred under argon atmosphere at rt for 2 h. The mixture was then diluted with CH_2Cl_2 and filtered through Celite. The precipitate was thoroughly washed with CH_2Cl_2, the filtrate was evaporated, and the crude material was purified on silica gel chromatography (50−80% EtOAc/hexane) to provide **42**α (180 mg, 39%) and **42**β (161 mg, 34%).

General Procedure for Glycosylation with Glycosyl Trichloroacetimidates. A flame-dried flask was charged with donor **40** (98 mg, 0.139 mmol), *N*-Fmoc-L-threonine benzyl ester (70 mg, 0.167 mmol), and 200 mg of 4 Å molecular sieves in a glovebag. The mixture was dissolved in 6 mL of dry CH_2Cl_2. The reaction mixture was cooled to −30 °C, trimethylsilyl triflate (14 μL, 0.5 equiv) was added, and the mixture was stirred until completion judged by TLC and then quenched with TEA. The mixture was then directly separated by flash chromatography on silica gel (50−80% EtOAc/hexane) to yield α-product **42**α (56 mg, 42%) and β-product **42**β (57 mg, 42%).

General Procedure for Glycosylation with Glycosyl Fluorides. A flame-dried flask was charged with *N*-Fmoc-L-threonine benzyl ester (28 mg, 1.5 equiv), Cp_2ZrCl_2 (12.9 mg, 0.044 mmol), $AgClO_4$ (18.2 mg, 0.088 mmol), and 200 mg of 4 Å molecular sieves in a glovebag. The mixture was cooled to −30 °C, and 1 mL of CH_2Cl_2 was added. Then, donor **39** (25 mg, 0.044 mmol) in 1 mL of CH_2Cl_2 was added dropwise, and the reaction was stopped after judged complete by TLC (1 h). Purification by silica gel chromatography yielded α-product **42**α (10 mg, 25%) and β-product **42**β (10 mg, 25%).

Compound 42α: $[\alpha]^{20}_{D}$ +61.44° (c 0.5, $CHCl_3$); IR (film) 3434, 3362, 3065, 2956, 2114, 1815, 1746, 1514, 1371, 1077 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, 2H), 7.67 (d, 2H), 7.47−7.33 (m, 9H), 5.78 (d, J = 9.4 Hz, 1H), 5.51 (d, J = 2.0 Hz, 1H), 5.24 (d, J = 12.3 Hz, 1H), 5.20 (d, J = 12.3 Hz, 1H), 5.12 (br s, 1H), 4.86−4.76 (m, 3H), 4.49−4.10 (m, 6H), 3.98 (dd, J = 10.4, 2.6 Hz, 1H), 3.88 (dd, J = 13.0, 6.9 Hz, 1H), 3.69 (dd, J = 10.6, 3.5 Hz, 1H), 2.26 (s, 3H), 2.18 (s, 3H), 2.16 (s, 3H), 2.02 (s, 3H), 1.29 (d, J = 7.0 Hz, 3H); ^{13}C NMR 100 MHz, $CDCl_3$) δ 170.2, 170.1, 169.8, 168.4, 156.5, 152.8, 143.7, 143.5, 141.0, 134.7, 132.4, 128.5, 128.4, 128.3, 127.5, 126.9, 124.9, 119.8, 100.2, 98.7, 77.1, 76.4, 72.2, 69.1, 68.1, 68.0, 67.6, 67.5, 67.1, 62.7, 62.1, 59.1, 58.6, 53.3, 46.9, 20.5, 20.4, 18.2. **42**β: $[\alpha]^{20}_{D}$ + 17.3° (c 0.64, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, 2H), 7.61 (d, 2H), 7.41−7.28 (m, 9H), 5.71 (d, J = 9.2 Hz, 1H), 5.35 (d, J = 2.9 Hz, 1H), 5.19 (d, J = 12.3 Hz, 1H), 5.11 (d, J = 12.3 Hz, 1H), 5.08 (t, J = 3.2 Hz, 1H), 5.00 (t, J = 3.4 Hz, 1H), 4.83−4.79 (m, 3H), 4.50−4.09 (m, 10H), 3.89 (dd, J = 11.4, 6.9 Hz, 1H), 3.62 (br t, 1H), 3.56 (t, J = 5.2 Hz, 1H), 3.36 (d, J = 10.4, 3.3 Hz, 1H), 2.19 (s, 3H), 2.18 (s, 3H), 2.12 (s, 3H), 2.03 (s, 3H), 1.32 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 170.4, 170.0, 169.8, 168.6, 156.8, 143.9, 143.7, 141.2, 135.3, 128.6, 128.4, 128.2, 127.6, 127.0, 125.2, 125.1, 120.0, 100.1, 99.9, 79.1, 74.9, 72.7, 72.4, 71.4, 68.4, 68.2, 67.7, 67.3, 62.5, 62.3, 61.9, 58.4, 47.1, 20.7, 20.6, 16.8; FAB HRMS calcd for (M + Na) $C_{47}H_{50}N_4O_{19}Na$ 997.2967, found 997.2961.

Compound 45. Glycal **44** (65 mg, 0.303 mmol) was dissolved in 1 mL of CH_2Cl_2 and treated with 6.06 mL of DMDO (0.06 M in acetone, 0.364 mmol) at 0 °C for 30 min. The solvent was removed *in vacuo* and placed under high vacuum for 1 h. The residue was dissolved in 2 mL of THF and cooled to −78 °C, and acceptor **19** (177 mg, 0.155 mmol) in 3 mL of THF was added followed by dropwise addition of 0.155 mL of $ZnCl_2$ (1.0 M in Et_2O, 0.155 mL). The reaction was allowed to warm to room temperature and stirred overnight. The reaction was quenched with saturated $NaHCO_3$, extracted with Et_2O, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by chromatography on silica gel (50−75% EtOAc/hexanes) to afford 128 mg (97%) of **45** as a white film: $[\mathbf{a}]^{20}_{D}$ +61.84° (c 0.25, $CHCl_3$); IR (film) 3425, 3064, 2920, 2112, 1809, 1740, 1513, 1237, 1042 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 9.0 Hz, 2H), 7.61 (d, J = 8.1 Hz, 2H), 7.55 (d, J = 8.0 Hz, 2H), 7.43−7.30 (m, 12H), 5.86 (d, J = 9.4 Hz, 1H), 5.56 (s, 1H), 5.21 (s, 2H), 4.93 (d, J = 3.3 Hz, 1H), 4.80 (d, J = 5.7 Hz, 1H), 4.48−4.44 (m, 2H), 4.25−4.23 (m, 3H), 4.25−4.03 (m, 9H), 3.90 (m, 2H), 3.61 (br s, 1H), 3.01 (br s, 1H), 2.12 (s, 3H), 1.30 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 170.1, 156.7, 153.5, 143.8, 143.6, 141.2, 137.4, 134.8, 129.0, 128.6, 128.5, 128.4, 128.1, 127.7, 127.0, 126.9, 126.4, 125.0, 199.9, 101.8, 100.8, 98.8, 76.1, 75.5, 75.4, 73.3, 69.2, 68.9, 68.7, 67.7, 67.2, 63.4, 62.2, 59.2, 58.7, 47.0, 20.6, 18.6; FAB HRMS calcd for (M + Na) $C_{48}H_{48}N_4O_{16}Na$ 959.2963, found 959.2927.

Fully Protected Threonine TF Antigen 46. Compound **45** (120 mg, 0.123 mmol) was treated with thiolacetic acid (5 mL, distilled three times) for 19 h at rt. The thiolacetic acid was removed with a stream of nitrogen, followed by toluene evaporation (three times). The crude product was purified by flash chromatography (80−100% EtOAc/hexane) to yield 103 mg (87%): $[\alpha]^{20}_{D}$ +90.18° (c 0.055, $CHCl_3$); IR

(film) 3324, 3064, 2954, 1815, 1747, 1674, 1527, 1372, 1228, 1043 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 2H), 7.65 (d, 2H), 7.43−7.32 (m, 7H), 5.90 (br d, J = 9.3 Hz, 1H), 5.76 (br d, 1H), 5.44 (br s, 1H), 5.20 (d, J = 12.0 Hz, 1H), 5.07 (d, J = 12.0 Hz, 1H), 4.97 (br d, J = 7.7 Hz, 2H), 4.81−4.76 (m, 3H), 4.48−4.41 (m, 4H), 4.18−3.90 (m, 8H), 2.20 (s, 3H), 2.16 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.29 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6, 170.5, 170.3, 170.1, 168.8, 156.5, 152.9, 143.6, 143.5, 141.1, 134.4, 128.7, 128.3, 127.6, 126.9, 124.9, 124.5, 119.9, 99.9, 75.0, 72.9, 72.3, 68.8, 68.2, 67.9, 67.5, 66.9, 62.9, 62.1, 60.2, 58.6, 48.7, 47.0 ,23.2, 20.8, 20.6, 20.5, 18.2, 14.0; FAB HRMS calcd for (M + Na) $C_{49}H_{54}N_2O_{20}Na$ 1013.3158, found 1013.3201.

Glycopeptide 48. Compound **47** (30 mg, 0.03 mmol) and 10 mg of 10% Pd/C were dissolved in 3 mL of MeOH under a hydrogen atmosphere (balloon) for 4 h. After consumption of starting material by TLC, the solution was filtered through Celite and concentrated to afford 27 mg (100%) of **48** as a white film: $[\alpha]^{20}_D$ +69.05° (*c* 0.2, $CHCl_3$); IR (film) 3333, 3064, 2938, 1817, 1746, 1642, 1528, 1372, 1226, 1044 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_6) δ 7.70 (d, 2H), 7.64 (t, 2H), 7.35 (t, 2H), 7.26−7.30 (m, 2H), 4.88−5.00 (m, 4H), 4.59−4.65 (m, 2H), 4.15−4.47 (m, 11H), 4.00 (dd, J = 11.0, 8.1 Hz, 1H), 3.92 (dd, J = 11.2, 3.0 Hz, 1H), 2.07 s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.95 (s, 3H), 1.18 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, MeOH-d_6) δ 173.6, 173.5, 172.4, 172.3, 172.2, 170.6, 159.1, 159.0, 155.2, 145.4, 145.2, 142.6, 128.9, 128.3, 128.1, 126.2, 126.1, 125.1, 121.1, 101.9, 100.9, 77.6, 76.7, 76.0, 74.9, 71.2, 70.6, 69.1, 67.7, 64.4, 63.5, 60.0, 59.9, 50.0, 48.5, 23.3, 21.0, 20.9, 20.8, 19.2; FAB HRMS calcd for (M + Na) $C_{42}H_{48}N_2O_{20}Na$ 923.2598, found 923.2692.

General Procedure D for Peptide Coupling with HOAt/HATU. Glycosyl amino acid **14** or **15** (1.0 equiv) and the peptide with a free amino gruop (1.2 equiv) were dissolved in CH_2Cl_2 (22 mL, 1 mmol). The solution was cooled to 0 °C, and IIDQ (1.15−1.3 equiv) was added (1 mg in ca. 20 mL of CH_2Cl_2). The reaction was then stirred at rt for 1−2 h. The mixture was then purified directly by chromatography on silica gel (3−5% MeOH/CH_2Cl_2) and subsequently deprotected.

General Procedure E for FMOC Deprotection with KF. A substrate (1 mmol in 36 mL of DMF) was dissolved in anhydrous DMF followed by addition of KF (10 equiv) and 18-crown-6 (catalytic amount). The mixture was then stirred for 48−60 h at rt until complete by TLC. Evaporation of DMF *in vacuo* was followed by flash chromatography on silica gel (7−10% MeOH/CH_2Cl_2). The resulting amine was then used directly in peptide coupling or capping with Ac_2O.

Glycopeptide 48. $[\alpha]^{20}_D$ +52.33° (*c* 0.7, $CHCl_3$); IR (film) 3249, 2942, 2865, 1748, 1684, 1370, 1243, 1034 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, 2H), 7.68 (t, 2H), 7.42−7.30 (m, 4H), 5.43 (d, J = 2.8 Hz, 1H), 4.92−4.83 (m, 3H), 4.57 (dd, J = 10.8, 6.3 Hz, 1H), 4.48 (dd, J = 10.8, 6.3 Hz, 1H), 4.39−4.09 (m, 11H), 3.94−3.89 (m, 2H), 3.21−3.15 (m, 2H), 3.02 (br t, 2H), 2.09 (br s, 6H), 2.06 (s, 3H), 2.00 (br s, 6H), 1.58 (t, J = 6.5 Hz, 2H), 1.45 (s, 9H), 1.20 (d, J = 6.3 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 170.5, 170.4, 169.9, 168.8, 157.0, 156.9, 152.8, 143.8, 143.7, 141.3, 127.7, 127.1, 125.1, 125.0, 120.0, 100.6, 100.1, 79.7, 77.7, 76.7, 72.6, 72.3, 69.0, 68.3, 68.2, 67.2, 63.0, 62.3, 58.7, 49.3, 47.2, 37.1, 36.0, 30.1, 28.4, 23.4, 20.8, 17.7; FAB HRMS calcd for (M + Na) $C_{50}H_{64}N_4O_{21}Na$ 1079.3961, found 1079.3955.

Glycopeptide 50. Compound **49** (70 mg, 0.03 mmol) was treated with 1.5 mL of TFA in 0.5 mL of CH_2Cl_2 for 30 min at rt. The reaction was concentrated and lyophilized with AcOH/*tert*-butyl alcohol (1:5). To the resulting white solid was added 18.3 mg (2.0 equiv, 0.06 mmol) of SAMA−OPfp in 2.5 mL of CH_2Cl_2 followed by the dropwise addition of DIEA (10.6 mL, 0.06 mmol). After 1 h at rt, the reaction mixture was concentrated and purified by silica gel chromatography (7−10% MeOH/CH_2Cl_2) to afford 63 mg of white film. This material was dissolved in a degassed solution of NaOMe in MeOH (pH ~10) under Ar for 2 h. The reaction was treated with Amberlyst-15 resin until pH ~5, and then filtered, concentrated, and purified via chromatography on RP-18 silica gel (degassed H_2O) to afford 36 mg (60%) of **50** as a fluffy solid after lyophilization. Further analysis was renounced to avoid dimerization of the thiol, and the material was stored under Ar at −78 °C: ^1H NMR (400 MHz, D_2O) δ 4.89−3.12 (m, ~60H), 2.13 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.69 (br m, 2H), 1.32 (d, J = 6.2 Hz, 3H), 1.29 (d, J = 5.8 Hz, 3H), 1.23 (d, J = 5.7 Hz, 3H); CI (NH_3) MS calcd for (M + Na) $C_{21}H_{27}N_3BrO_{14}Na$ 1611.8, found 1611.8.

Vaccine Preparation. **27**−KLH and **27**−BSA vaccines were prepared as described previously for the MUC1−KLH conjugate vaccine.[45] The yield of this procedure was generally around 10%. Unreacted Tn(c) was removed by a molecular cutoff filter (MW 30 000, Centriprep, Amicon Inc., Beverly, MA). The samples was filtered through a 0.22 μm filter under sterile conditions. The protein content was determined using the BioRad protein assay and the carbohydrate content by a HPAEC-PAD assay. The epitope ratio of **27**−KLH and −BSA was 317:1 and 7:1, respectively. The resulting more effective conjugation to KLH versus BSA may reflect uncertainties in the quality of the reagents employed for the latter. The aliquoted vaccine was stored at 4 °C.

Immunization Protocol. Groups of mice (female CB6F1 mice, Jackson Laboratory, Bar Harbor, ME) were immunized subcutaneously five times (weeks 0, 1, 2, 7, and 20) with 10 μg of **30** alone dissolved in intralipid, 10 μg of **30** alone dissolved in intralipid plus 20 μg of QS-21, and **27**−KLH or **27**−BSA (containing 3 μg of synthetic Tn(c) trisaccharide) plus 20 μg of QS-21. Mice were bled 10 days after the third, fourth, and fifth vaccinations and, sera were separated and stored at −30 °C.

Serological Analysis. The serological response was analyzed by several serological methods.

(1) ELISA. Enzyme-linked immunosorbent assay (ELISA) was used to determine the titer of antibodies against Tn(c)−pam as described previously.[44] Serially diluted antiserum was added to wells coated with antigen (0.1 μg) and incubated for 1 h at room temperature. Goat anti-mouse IgM or IgG conjugated with alkaline phosphatase served as secondary antibodies. Absorbance was measured at 414 nm. The antibody titer was defined as the highest serum dilution showing an absorbance of 0.1 or greater than that of normal mouse sera.

(2) Flow Cytometry. Cell surface reactivity of these antibodies was assayed by flow cytometry on Tn(c) positive LS-C cells and Tn(c) negative LS-B cells.[45] Single cell suspensions of 2 × 10^5 cells/tube were washed in PBS with 3% fetal calf serum and 0.01 M NaN_3 and incubated with 20 μL of 1:20 diluted antisera or mAb IE3 for 30 min on ice. After the cells were washed twice with 3% FCS in PBS, 20 μL of 1:15 goat anti-mouse IgM labeled with fluorescein−isothiocyanate (FITC) was added. The solution was mixed and incubated for 30 min. After the cells were washed, the positive population and mean fluorescence intensity of stained cells were analyzed by flow cytometry (EPICS-Profile II, Coulter, Co., Hialeah, FL) as described.[46]

(3) Complement Dependent Cytotoxicity (CDC). The ability of these sera to mediate complement lysis was assessed by a chromium release complement dependent cytotoxicity assay against LS-C cells (6). Complement dependent cytotoxicity was assayed at a serum dilution of 1:10 with LS-C cells by a 4 h chromium-release assay as previously described. All assays were performed in triplicate. Controls included cells incubated only with culture medium, complement, antisera, or mAb IE3. Spontaneous release was the europium released by target cells incubated with complement alone. Percent cytolysis was calculated according to the formula specific release (%) = experimental release − spontaneous release/maximum release − spontaneous release × 100

Inhibition Assay. Antisera at 1:1500 dilution or mAb IE3 at 0.1 μg/mL were mixed with various concentrations of structurally related and unrelated carbohydrate antigens. The mixture was incubated at room temperature for 30 min, and transferred to an ELISA plate coated with Tn(c)−pam. ELISAs were performed as described above. Percentage inhibition was calculated as the difference in absorbance between the uninhibited and inhibited serum.

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ELISAs were also performed with sera that had been inhibited (absorbed) by incubation with LS-C or LS-B cells. For this assay 5×10^5 cells were incubated with sera for 1 h and the cells removed by centrifugation. ELISA was performed as described above.

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Supporting Information Available: ^{1}H and ^{13}C spectra for compounds **5**, **7**, **9**, **14**−**19**, **21**, **23**, **25**, **30**, **35**−**38**, **40**β, **41**α, **42**α, and **45**−**47**, ^{1}H spectra for compounds **32**, **39**α,β, **40**α, and **50**, and experimental details for compounds **14**−**17**, **20**−**23**, **35**, **41**α, **41**β, and **49** (60 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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A Broadly Applicable Method for the Efficient Synthesis of α-*O*-Linked Glycopeptides and Clustered Sialic Acid Residues

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Contribution from the Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10021, and Department of Chemistry, Columbia University, New York, New York 10027

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Contribution from the Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10021, and Department of Chemistry, Columbia University, New York, New York 10027

Received September 17, 1998

Abstract: The total syntheses of complex sialylated cell-surface antigens have been accomplished. The target systems include 2,3-STF, STn, 2,6-STF, and glycophorin antigens. In addition, an α-*O*-linked serine glycoside of an entire Lewis blood group (Y) antigen has been assembled.

Introduction

As part of a multidisciplinary program targeted to the enlistment of the human immune system for recognition of various tumors and for mounting a clinically helpful response, we have directed our attention to the mucin class of glycoproteins. Mucins represent a family of cell-surface glycoproteins often associated, in aberrant glycoforms, with tumors of epithelial tissues.[1] In the first phase of our investigations, reported in an earlier publication, we have demonstrated that the trimeric clusters of Tn and TF glycoepitopes, conjugated as such to the Pam_3Cys lipophilic immunostimulant[2] or to KLH (keyhole limphet hemocyanin) immuno-protein carrier,[3] are immunogenic as judged by antibody production. Furthermore, the clustered motifs provoke robust production of antibodies, with promising cell-surface reactivity for those tumors expressing the respective antigen. Seemingly, our fully synthetic vaccine constructs, with trimeric arrangement of Tn epitopes, are able to mimic the cell-surface presentation of mucin *O*-linked antigens.[3] That proof-of-principle study provided particularly strong incentives to develop generally applicable schemes for the preparation of even more complex members of this class. In particular, we focused on the development of synthetic methodology to reach sialylated members of the TF family of mucin antigens including the glycophorin family. The state of sialylation (usually accomplished intracellularly by sialyltransferases or by de-sialylation within a cell by sialidases) is a critical determinant in cell-surface recognition of glycoproteins. In stepping up the complexity level of our goals, we would be confronting a long-standing problem in oligosaccharide and glycoconjugate synthesis. The crux of the difficulty has been the problematic character of synthesizing carbohydrate domains *O*-linked to the key amino acids, serine and threonine, with strong stereochemical control in the formation of the α-glycosidic linkage. In previous publications we have shown that the conduct of the glycosylation in the context of a fully assembled complex glyco-domain (such as a glycal) is fraught with considerable uncertainty as to the eventual α/β ratios.[2,4] While success was achieved in a few cases, seemingly small structural variations in fact profoundly affect the quality of the various steps in the progression from terminal glycal to *O*-linked serine or threonine glycoside in ways that are difficultly interpretable.[2] After much study of many cases, we are unable to provide a generally reliable protocol that would deliver the required serine or threonine glycoside to mature glycodomains with high α-selectivity to *any substrate of our choosing*. Given the potentially great importance of the synthesis of complex cell-surface molecular mimics in which serine or threonine residues are omnipresent in α-linkages, we viewed this uncertain situation as a serious detriment to progress. Herein we report a remarkably general solution to reach our goal systems. The method has been tested under stringent challenges such as are involved in the stereoselective syntheses of particularly complex and potentially invaluable cell surface molecular mimics. The assembly logic we describe is that of a "cassette" modality rather than reliance on a maximally convergent approach cited above. In the traditional regime,[4] a full glycodomain is assembled. An α-linkage to serine or threonine is then fashioned at the reducing end *of the fully mature domain*. Aside from the unpredictable glycosylation ratios in any new case, another disadvantage of this strategy is that difficulties in the yield or stereoselectivity during installation of the α-*O*-linked serine or threonine are borne out by the full glycodomain. Furthermore, in the traditional approach the protecting groups throughout the domain must be compatible with installation of the glycosyl donor functionality, and with the coupling step to the serine or threonine. Most serious is the nonreliability of the stereochemistry of this type of glycosylation in any new case. *In our new approach, a terminal GalNAc residue bearing an in-place serine or threonine is used as a generalized acceptor to be appended to a suitable donor linkage.*[5] Hopefully we would be facing less problematic glycosylation challenges associated with the saccharide−saccharide coupling in our assembly process. Ideally, such couplings would be conducted with a suitably

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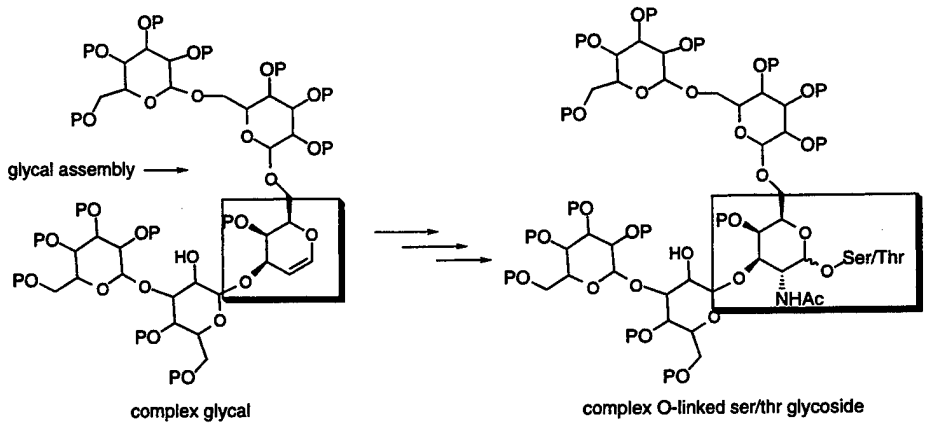
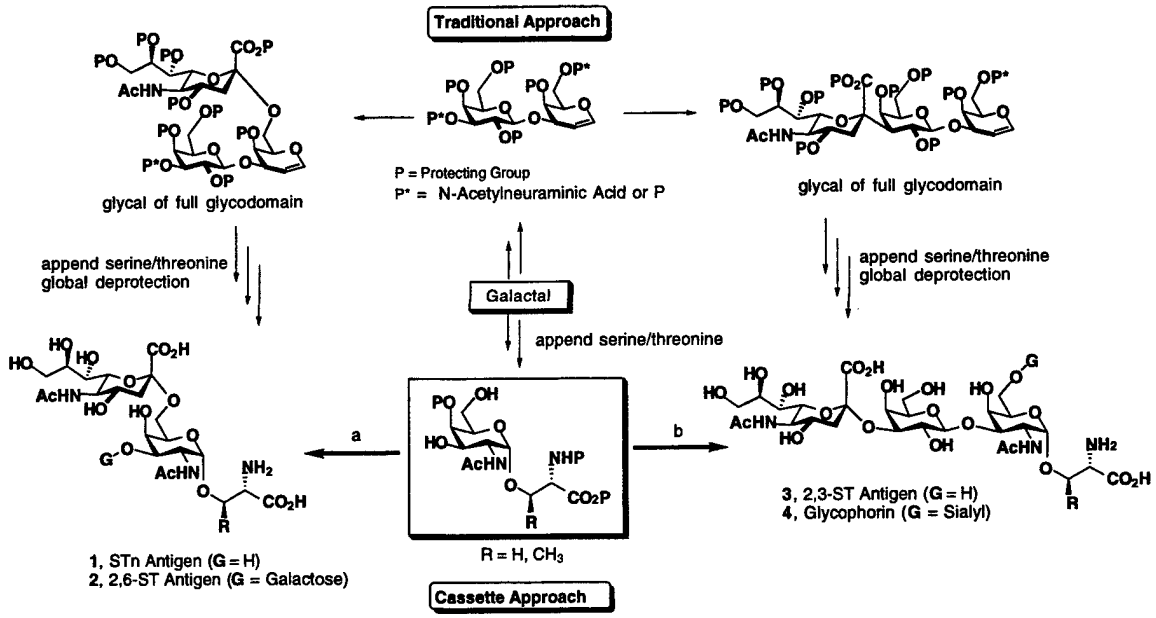


Figure 1.



a) append sialic acid and galactose, b) append sialic acid and sialyllactose to the cassette.

Figure 2.

protected serine or threonine residue (properly protected) *in place* at the "reducing end" of the acceptor. Figure 2 contrasts both approaches drawing particular attention (see bold arrows) to the bond being established between the cassette and the rest of the domain.[6]

Four distinct subgoals had to be achieved to implement the new program. First we had need to synthesize acceptor systems (cassettes) with α-*O*-linked serine or threonine residues, in place, in the context of GalNAc or latent GalNAc units. Moreover, these GalNAc units must bear differentiated or readily differentiable acceptor sites. Furthermore, the actual glycosylation with the acceptor cassette bearing the in-place *O*-linked serine or threonine had to proceed smoothly. Finally, global deprotection of the fully assembled construct must be achievable without undermining the stability of the potentially fragile linkage connecting the carbohydrate and amino acid domains.

In the work described herein, we set about to field test our notions regarding these challenges in the context of synthesizing sialylated antigens which are α-*O*-linked to peptides. Clearly, the inclusion of these sialic acid residues represents a significant extension in the complexity level of the undertaking. There was a clear rationale for undertaking such additional chemical challenges. Thus, the STn antigen **1**, which contains a sialic acid residue at position 6 of galactose is abundantly expressed in major epithelial tumors of the breast, ovary, colon, and stomach.[7] The presence of sialic acid moieties at either the 6-*O* position or the 3′-*O* position of the TF disaccharide is found in the 2,6-STF[8] and 2,3-STF[9] antigens **2** and **3**, respectively. The presence of sialic acid moieties on both the 6-position of the GalNAc and the 3-position of the galactose is the distinctive feature of the parent member of the family, glycophorin **4**. This motif is found in the context of a major erythrocyte membrane

(5) In practice since the assembly state of the 2,6-STF antigen (**2**) worked reasonably well by convergent glycal assembly, we did not resynthesize this goal structure through full implementation of the cassette method with two separate donors.

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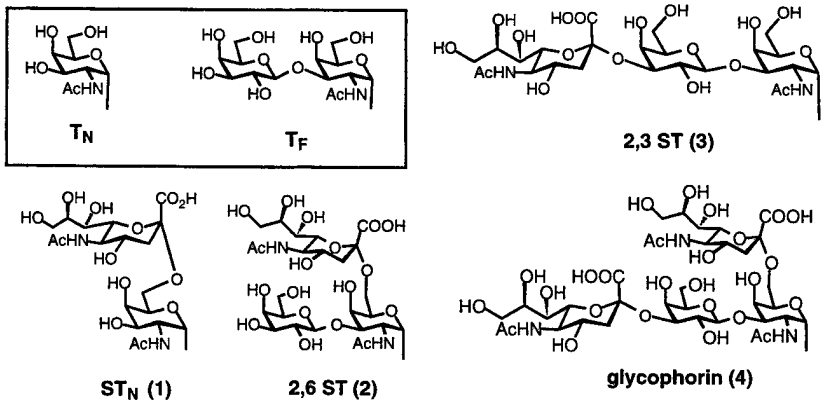
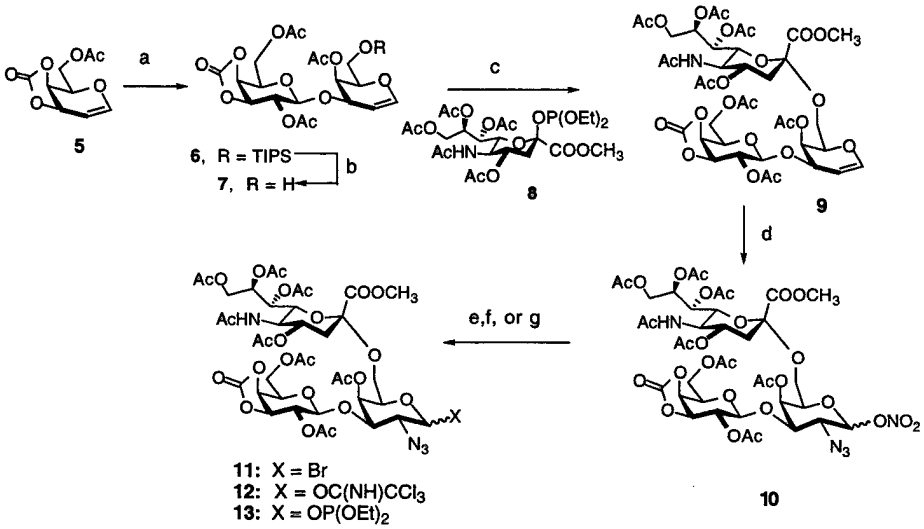


Figure 3.

Scheme 1



Reagents: (a) 1. DMDO, CH_2Cl_2; 2. 6-*O*-TIPS-galactal, $ZnCl_2$, -78 to 0 °C; 3. Ac_2O, Et_3N, DMAP, 75%.; (b) TBAF, AcOH, THF, 80%; (c) **8**, TMSOTf, THF:toluene (1:1), -60 to -45 °C, 84%, α:β 4:1; (d) NaN_3, CAN, CH_3CN, -15 °C, 60%; (e) LiBr, CH_3CN, 75%; (f) 1. PhSH, $^iPr_2NEt$, CH_3CN, 82%; 2. Cl_3CCN, K_2CO_3, CH_2Cl_2, 80%; (g) 1. PhSH, $^iPr_2NEt$; 2. $ClP(OEt)_2$, $^iPr_2NEt$, THF, 72%

glycoprotein.[10] Moreover, the presence of the 2,3-STF antigen **3** on breast tumors has been demonstrated,[9] and the expression of the 2,6-STF antigen **2** on cells of myelogenous leukemia has also been identified.[8] Our target structures are identified in Figure 3. From a chemical standpoint, the effective introduction of sialic acid residues into glycoconjugates has been a challenge for many years.[11] Careful considerations, as to which sialylation protocols would prove to be workable with either a glycal linkage in place or with α-*O*-linked glycosyl amino acid acceptors, would be a critical element of the synthetic design. In the studies described below, the two approaches discussed above were considered for accommodating the inclusion of sialic acid residues: (1) assembly of an entire glycodomain in the form of an advanced glycal, followed by the attachment of the amino acid; (2) an alternative approach wherein a "cassette", comprising a reactive acceptor having the *O*-linked amino acid attached to the GalNac residue, is united with a donor already equipped with a sialic acid. Such an approach would provide a suitably protected precursor to target structures **1**–**4** (Figure 2).

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Results and Discussion

1. Synthesis of 2,6-STF Glycosyl Serine/Threonine via the Modular Glycal Approach. To construct the glycosylated amino acid building blocks corresponding to the 2,6-STF[12] antigen for incorporation into a peptide backbone, we turned to the practice of glycal assembly (Scheme 1).[13] In the event, 6-*O*-TIPS galactal was coupled with compound **5**, to provide disaccharide **6** in 75% yield. After deblocking of the 6-*O*-TIPS moiety with buffered TBAF–HOAc, sialylation was attempted with various sialic acid donors. The diethyl phosphite donor[14] **8** proved to be most effective and afforded trisaccharide **9** in 84% yield as a separable 4:1 α:β mixture of anomers. Other donors such as a sialyl chloride[15] gave similar yields but with poorer sialic anomer selectivity.

(12) For previous synthesis of 2,6-STF, see (a) Iijima, H.; Ogawa, T. *Carbohydr. Res.* **1989**, *186*, 95. (b) Qiu, D.; Koganty, R. R. *Tetrahedron Lett.* **1996**, *37*, 595. (c) Qiu, D.; Koganty, R. R. *Tetrahedron Lett.* **1997**, *38*, 961.

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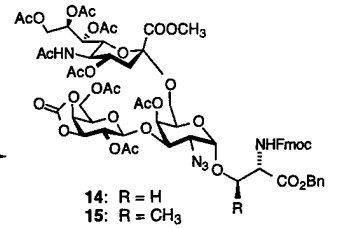
Table 1.

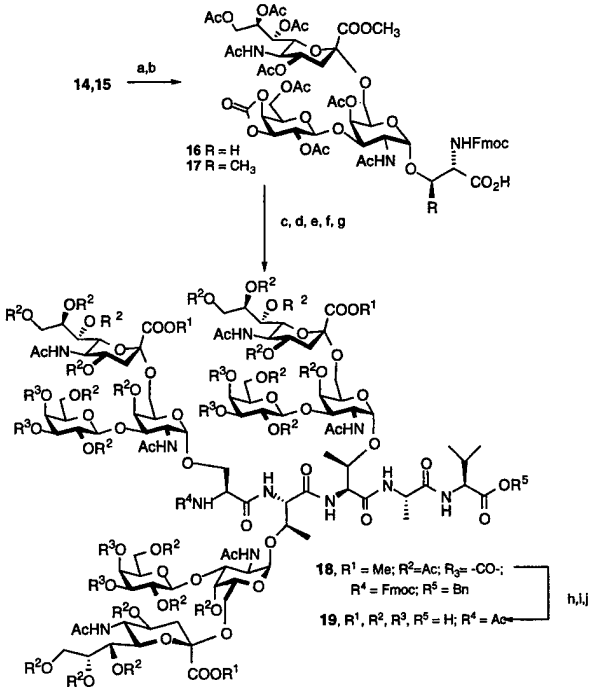


X	Catalyst / Promoter	R = H (14) α : β (%)	R = CH_3 (15) α : β (%)
- Br (11α)	$AgClO_4$ (1.5 eq), CH_2Cl_2, rt	2.6 : 1 (70%)	α only (74%)
- O(CNH)CCl_3 (12β)	BF_3•OEt_2 (0.5 eq), THF, -30° C	12 : 1 (65%)	α only (63%)
- O(CNH)CCl_3 (12α,β 1:1))	BF_3•OEt_2 (0.5 eq), THF, -30 °C	4 : 1 (66%)	α only (60%)
- OP(OEt)$_2$ (13α,β 1:1)	BF_3•OEt_2 (0.5 eq), THF, -30 °C	30 : 1 (30%)	——

We now had to deal with the introduction of the *O*-linked amino acid to a donor ultimately derived from glycal **9**. The resultant galactal underwent smooth azidonitration[16] resulting in installation of the latent galactosamine for eventual glycosylation of serine or threonine. Conversion of compound **10** to a number of candidate donor linkages was achieved. Direct displacement of the anomeric nitrate to the α-bromide with LiBr/CH_3CN furnished anomeric bromide **11** in 75% yield. Reduction of the nitrate to the hemiacetal (PhSH, $^iPr_2NEt$) could be followed by conversion to the trichloroacetimidate **12** in 80% yield.[17] Alternatively treatment of the hemiacetal with chlorodiethyl phosphite gave the unstable phosphite donor **13**. Both **12** and **13** were obtained as mixtures of anomers in a 1:1 ratio. The results of attempts to glycosylate *N*-Fmoc-protected serine and threonine benzyl ester with donors **11**−**13** are shown in Table 1. With these donors, the coupling to protected threonine acceptors occurred with complete stereoselectivity, exclusively yielding the desired α-linked product **15**. While not proceeding with equivalent stereocontrol, the coupling of donors **12** and **13** with protected serine acceptors still provided the desired α-*O*-linked anomer as the major products in the indicated yields. The diethyl phosphite donor **13** afforded the highest selectivity. Noteworthy was the fact that a mixture of **12**α and **12**β gave a lower selectivity than pure **12**β. We had previously found that related donors where the 6-*O*-sialic acid in **11**−**13** is replaced by an acetate, the selectivity for both serine and threonine drops off to 2:1 α:β at best.[2] However, the types of difficulties as exemplified in the glycophorin synthesis (vide infra) in generalizing serine or threonine glycosylation ratios led us to favor the cassette approach. At this stage, our initial objective was achieved: a rapid, efficient glycodomain assembly via glycal logic, with resident protecting groups suitable for subsequent peptide coupling steps, and mild basic deprotection to conclude the synthesis was realized.[4] We first describe the construction of the 2,6-STF antigen motif.

2. Assembly of CD43-Derived Glycopeptides with Clustered 2,6-STF Epitopes. Having accomplished the synthesis of the 2,6-STF antigen, albeit in a nongeneral fashion, we set our sights on clustering such a substructure to produce a mimic portion of the CD43 glycopeptide.[18] The azido groups of trisaccharides **14** and **15** were reduced with thiolacetic acid[19] (78% yield) followed by quantitative hydrogenolytic removal

Scheme 2



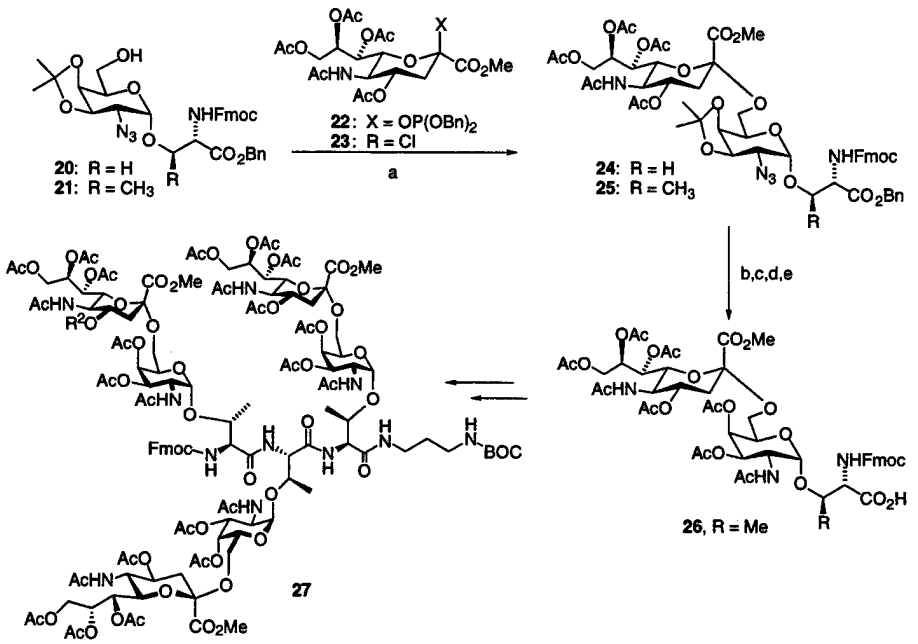
Reagents: (a) AcSH, 78%; (b) H_2, Pd/C, MeOH/H_2O, 100%; (c) H_2N-Ala-Val-OBn, IIDQ, CH_2Cl_2, 85%; (d) KF, DMF, 18-crown-6, 95%; (e) **17**, IIDQ, 87%; (f) KF, DMF 18-crown-6, 93%; (g) **15**, IIDQ, 90%; (h) 1. KF, DMF, 18-crown-6; 2. Ac_2O, CH_2Cl_2 (i) H_2, Pd/C, MeOH/H_2O; (j) NaOH, MeOH/H_2O, pH 10-10.5, 80%

of the benzyl ester, to afford **16** and **17**. These compounds served as suitable building units for the glycopeptide assembly. The glycopeptide backbone was elaborated from the carboxy to amino terminus direction (Scheme 2). Trisaccharide **17** was coupled to Ala-Val-benzyl ester (IIDQ), and iterative peptide coupling steps between the N-terminus of the peptide and the protected glycosyl amino acid, gave the desired pentapeptide **18** in high yield (average 85% for each coupling step). Finally, the carbamate linkage was deprotected and the amine capped with an acetyl group. After global deprotection (NaOH−H_2O, MeOH), the desired CD43 glycoprotein N-terminus **19** containing the clustered 2,6-STF epitopes was obtained in 80% yield and high purity as determined by reverse-phase HPLC (MeOH/H_2O, C18, 215 nm). This cluster is currently being readied for immunoconjugation and study.

3. Synthesis of STn Glycosyl Serine/Threonine via the Cassette Approach. In our synthesis of the blood-group determinant F1α,[20] it had been shown that lactosylation of a building block containing an acceptor hydroxyl at the 6-*O*-position could be effected. This strategy provided a fully protected precursor to free F1α. It was hoped that, in a related way, we could gain access to the STn antigen with high synthetic economy using protected glycosyl amino acid derivatives (see cassettes **20** and **21**) and a sialic acid-containing donor as a counterpart of the lactosylation system.[21] A synthesis of the STn monomer and its incorporation into a peptide was recently reported by Kunz.[22] Indeed, when either of the glycosylated serine or threonine cassettes **20** or **21**, respectively, were exposed

(16) Lemieux, R. U.; Ratcliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244.
(17) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Biochem.* **1994**, *50*, 84−123.
(18) Carlstedt, I.; Davies, J. R. *Biochem. Soc. Trans.* **1997**, *25*, 214.
(19) (a) Bieldfeldt, T.; Peters, S. Meldal, M.; Bock, K.; Paulsen, H. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 857. (b) Rosen, T.; Lico, I. M.; Chu, D. T. W. *J. Org. Chem.* **1988**, *53*, 1580.
(20) Chen, X.-T.; Sames, D.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 7700−7709.
(21) For previous synthesis of STn via glycosylation of a disaccharide see: (a) Iijima, H.; Ogawa, T. *Carbohyr. Res.* **1988**, *172*, 183. For a related example using a similar type of cassette, see (b) Liebe, B.; Kunz, H. *Tetrahedron Lett.* **1994**, *35*, 8777. (c) Elofsson, M.; Salvador, L. A.; Kihlberg, J. *Tetrahedron* **1997**, *53*, 369. (d) Yule, J. E.; Wong, T. C.; Gandhi, S. S.; Qiu, D.; Riopel, M. A.; Koganty, R. R. *Tetrahedron Lett.* **1995**, *36*, 6839.
(22) Liebe, B.; Kunz, H. *Helv. Chim. Acta* **1997**, *80*, 1473.

Scheme 3

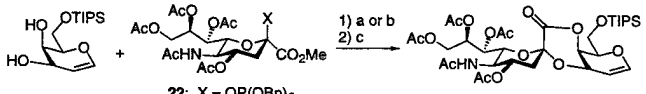


Reagents: a) TMSOTf, THF, 4Å mol sieves, -40 °C (R = Me, X = OP(OBn)2, 37%; AgOTf, DTBP, $CaSO_4$, THF, -78 °C (R = H, X = Cl, 50%); b) AcSH, pyr, 87%; c) 80% AcOH; d) Ac_2O, Et_3N, DMAP, CH_2Cl_2, 84% (two steps); e) H_2, Pd/C, MeOH/H_2O, 100%.

to a phosphite donor **22** or chloride donor **23**, the desired disaccharides **24** and **25** were obtained (Scheme 3). The stereochemistry of the addition was determined to be α from the chemical shift pattern at C3 in the ^1H NMR spectrum of the sialyl residue. In the case of **25**, although the overall α:β selectivity was 4:1, separation of the anomers proved to be tedious and the yield of pure α-adduct was only 37%. Kunz has made similar observations in both selectivity and yields, using sialyl xanthate donors.[23] Noteworthy, but not helpful, was the fact that sialylation using CH_2Cl_2 as solvent instead of THF led to the exclusive formation of the undesired β-anomer. In preparation for the impending peptide coupling, a short synthetic sequence was necessary. The phase commenced with the reduction of the azide linkage of **25** (AcSH, pyridine, 87%). Removal of the acetonide, reprotection as the acetates, and hydrogenolysis of the benzyl ester provided the compound **26**, necessary for clustering in 84% two-step yield. An iterative peptide coupling process, as employed above for the 2,6-STF series, afforded **27**. This tripeptide is differentially protected on each terminus to enable further elaboration. Conjugation of **27** to an immunostimulant or suitable derivative is now underway.

4. Synthesis of 2,3-STF Glycosyl Threonine via the Cassette Approach. Given the success of the synthesis and clustering of the 2,6-STF and STn antigens, we turned our attention toward the 2,3-STF antigen, which has been associated with human breast cancer.[8] The chemical synthesis of 2,3-STF by a similar approach has been recently reported[24] as has a fully enzymatic procedure.[25] Initially, as implemented in the 2,6-STF synthesis, it was our strategy that construction of the trisaccharide portion, would be followed by azidonitration and subsequent attachment of the full carbohydrate donor to a protected serine or threonine acceptor. Initial efforts at preparing trisaccharide

Scheme 4



Reagents: (a) TMSOTf, THF, -40 °C (X = OP(OBn)2); (b) AgOTf, DTBP, CH_2Cl_2, -78 °C (X = Cl); (c) DBU, 0 °C, 40-50%

met with many complications. A serious problem was the difficult sialylation at the 3′-*O*-position of a suitably protected disaccharide glycal (TF core). This approach was also plagued by extremely poor yields and low selectivities.[26]

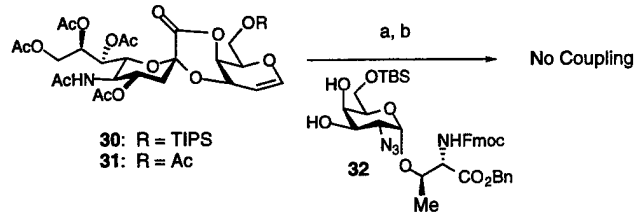
As an alternative approach, we sought to apply the cassette technology.[2,6] The goal would involve coupling of the appropriately protected cassette with a suitable donor derived from a disaccharide such as **30**. Thus, sialylation of compound **29** was carried out using phosphite donor **22** mediated by TMSOTf,[13] or alternatively with donor chloride **23** mediated by AgOTf.[27] The reaction mixture was then treated with DBU at 0 °C for 1 h to afford compound **30** in 40−50% yield for the two steps.[26,28]

The cassette coupling was attempted using the strategy previously employed by us for the TF antigen.[2] Thus, treatment of **30** with DMDO was followed by exposure to acceptor **32** in the presence of $ZnCl_2$. However, no coupling was observed. It was thought that the TIPS group in **30** was again too demanding as a steric presence. However, even with an acetate at the corresponding position (see **31**), no coupling was observed.

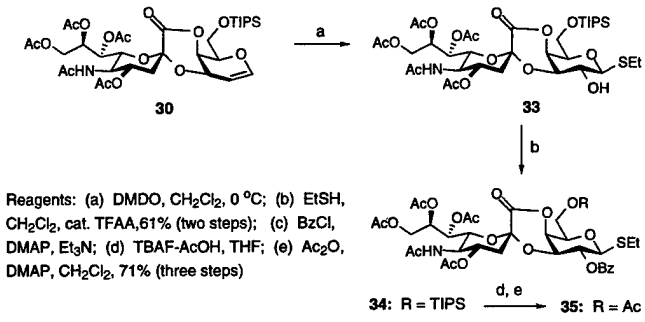
In previous studies, we and others have shown that thioethyl glycosides, suitably protected at C2 (pivalate, benzoate), can serve as excellent β-glycosidation donors in a variety of glycosylation reactions.[29] We were intrigued with the possibility of a convergent coupling of an inactivated thio donor such as compound **33** with acceptor **32**. In the event, treatment of **30**

(23) Liebe, B.; Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 618.
(24) Nakahara, Y.; Nakahara, Y.; Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1997**, *38*, 7211.
(25) Synthesis: Kren, V.; Thiem, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 893.
(26) Woo, J. W.; Kuduk, S. D.; Danishefsky, S. J. Unpublished results.
(27) Gervay, J.; Peterson, J. M.; Oriyama, T.; Danishefsky, S. J. *J. Org. Chem.* **1993**, *58*, 5465.
(28) Ercegovic, T.; Magnusson, G. *J. Org. Chem.* **1995**, *60*, 3378.

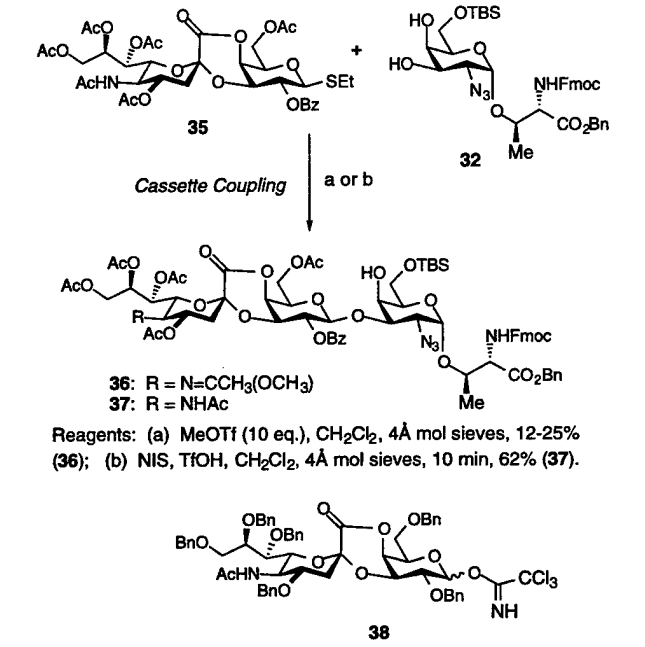
Scheme 5



Scheme 6



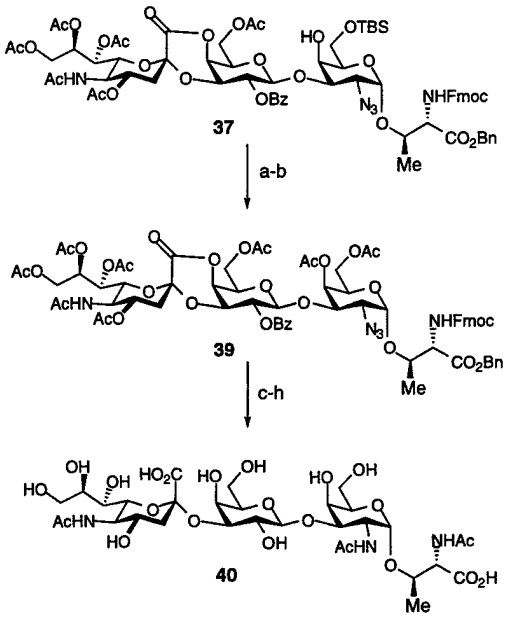
Scheme 7



with DMDO, followed by epoxide opening with ethanethiol in the presence of catalytic trifluoroacetic acid, provided **33** in 61% yield, to serve as the thiodonor. Benzoylation at C2 afforded **34**, and subsequent conversion of the 6-*O*-TIPS group to acetate furnished the projected donor **35** in 71% yield with only a single purification step. The critical coupling step was at hand.

Glycosylation of **34** with acceptor **32** was first investigated using our previously developed conditions (MeOTf, DTBP).[29a] No coupling was observed. Our first response to this setback was to replace the 6-*O*-TIPS group with an acetoxy function as in donor **35**, which would hopefully be less encumbered based upon our previous observations with TF.[2] Although some

Scheme 8



coupling products were obtained, the reaction proved quite inefficient and gave generally poor yields (ca. 25%). Another consequence of using methyl triflate as the promoter, was the formation of the compound **36** at the C-5 amide of the sialic acid. Cleavage of the methyl group required an additional hydrolytic step.

After surveying a variety of alternative coupling conditions, it was found that recourse to the Fraser-Reid *N*-iodosuccinimide–triflic acid promoter system[30] afforded compound **37** in ca. 60% yield with good reproducibility. Of course, in the absence of a methylating potential in the promoter, no imidate byproduct was observed.

A somewhat related approach had been previously employed by Ogawa using trichloroacetimidate **38**.[31] It should be noted here that thiodonor **35** was highly unactivated due the presence of the acetates, while donor **38** was an electron rich perbenzylated system. Thus, although we have exploited a "disarmed"[32] glycosyl donor in the case of thiodonor **35**, the use of the NIS/TfOH[30] promoter system was successful toward 2,3-STF. We have also extended this approach to other "disarmed" glycosyl donors (vide infra). Global deprotection starting with **37** to expose the free antigen was accomplished in a straightforward manner (Scheme 8). Hence, removal of the 6-*O*-TBS group on the GalNAc residue was achieved with 1 N HCl. Acetylation of the free hydroxyl groups provided trisaccharide **39** with all resident hydroxyl groups protected as esters in 85% yield. At this stage the stereochemistry of the NIS coupling product was determined to be β by COSY and HETCOR experiments. Reduction of the azide with thiolacetic acid proceeded in 56% yield. Deprotection of the amino acid portion was effected by

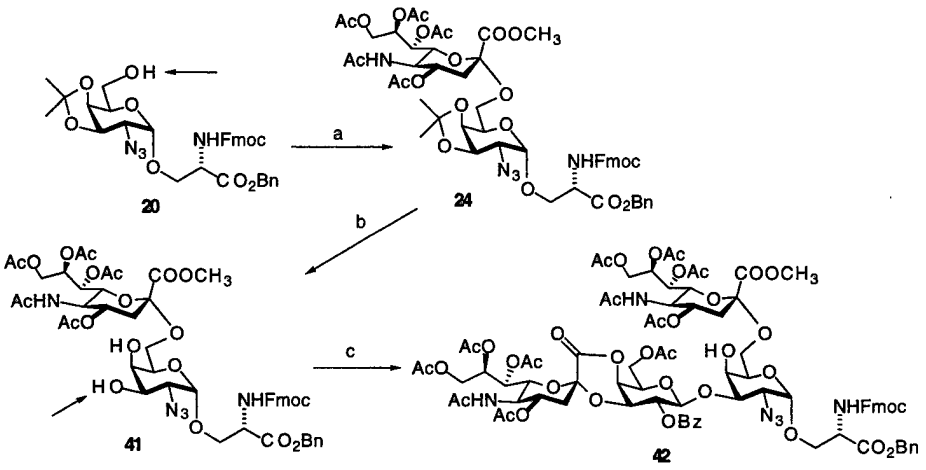
(29) (a) Seeberger, P. H.; Eckhardt, M.; Gutteridge, C. E.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 10064. (b) Garegg, P. J.; Hentrichson, C.; Norberg, T. *Carbohydr. Res.* **1983**, *116*, 162. Reviews on thioglycosides: (c) Fujed, P.; Garegg, P. J.; Lonn, H.; Norberg, T. *Glyconconjugate J.* **1987**, *4*, 97. (d) Toshima, K.; Tatsuta, K. *Chem. Rev.* **1993**, *93*, 1503.

(30) Konradsson, P.; Udodong, U. E.; Fraser-Reid, B. *Tetrahedron Lett.* **1990**, *31*, 4313.

(31) Nakahara, Y.; Nakahara, Y.; Ito, Y.; Ogawa, T. *Tetrahedron Lett.* **1997**, *38*, 7211.

(32) (a) Mootoo, D. R.; Konradsson, P.; Udodong, U. E.; Fraser-Reid, B. *J. Am. Chem. Soc.* **1988**, *110*, 5583. (b) Halcomb, R. L.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1989**, *111*, 6661. (c) Veeneman, G. H.; van Boom, J. H. *Tetrahedron Lett.* **1990**, *31*, 275. (d) Veeneman, G. H.; van Leeuwen, S. H.; van Boom, J. H. *Tetrahedron Lett.* **1990**, *31*, 1331.

Scheme 9



Reagents: (a) **23** (X= Cl), AgOTf, $CaSO_4$, DTBP, THF, -78°C; (b) AcOH, 12 hr; (c) **35**, NIS, TfOH, CH_2Cl_2, 4Å mol sieves, 10 min, 53%

carbamate removal (morpholine), and capping of the free amine was achieved by acetylation. Subsequent hydrogenolysis of the benzyl ester, hydrolysis of the acetates with sodium hydroxide in aqueous methanol, and removal of the 2′-*O*-benzoate (NaOMe, MeOH, 65 °C) provided *N*-acetyl-2,3-STF **40**. While the yield of the global deprotection sequence was only 44% overall, some of the loss could be accounted for via β-elimination of the amino acid under the harsh basic conditions.[33] Clustering of the antigen and immunological evaluation can be realized in an analogous fashion as for the 2,6-STF and STn glycopeptides and will be reported in due course.

5. Synthesis of a Glycophorin Glycosyl Threonine Precursor via a Modified [2 + 2] Cassette Approach. Having successfully accessed the other members of the ST antigen family, we directed our attentions toward an even more complicated venture. The glycophorin antigen is encountered on a major glycoprotein found on the human erythrocyte membranes.[34] To date, only Ogawa has reported a total synthesis of an *O*-linked glycophorin. However, the Ogawa routes were plagued by poor selectivity in the amino acid glycosylations,[35] or by recourse to a series of mono-glycosylations, which required significant manipulating of protecting groups.[36] For application of our methodology, a properly *O*-linked disaccharide acceptor would be required. For this purpose we required an undifferentiated acceptor such as **41**. In this undertaking, in an overall sense (see arrows) we would be using cassette **20** with two different donors in a properly timed way. Accordingly, sialylation of **20** with sialic acid donor **23** afforded **24**, followed by acetonide cleavage using AcOH to yield compound **41** in 48% yield for the two steps. We hoped that the glycosylation (i.e. sialylgalactosylation) would occur at C3 of the galactose segment of **41** (see arrow) which is equatorial. In the event, coupling of **41**, prepared as shown, with **35** using the Fraser-Reid conditions[30,32] afforded the protected *O*-linked tetrasaccharide **42** in 53% yield.

5. Synthesis of a Le^y Hexasaccharide Glycosyl Serine Precursor via a Modified [5 + 1] Cassette Approach. As a further extension of this new methodology, we examined an even more ambitious "disarmed" glycosylation[30] process related to an ongoing Le^y project in our laboratory.[37] In this regard, we were interested in producing α-*O*-linked Le^y structures. One such construct was hexasaccharide **45**, which could be obtained in a [5 + 1] cassette coupling with a pentasaccharide donor. In the event, thio-donor **43** and cassette **44** were coupled via NIS/TfOH in an exemplary 79% yield. *It will not go unnoticed that **45** constitutes the basic substructure by which a Lewis Y blood group determinant can be presented in the context of an advanced cell-surface mimic.* We are currently exploiting this remarkable cassette coupling method.

Summary

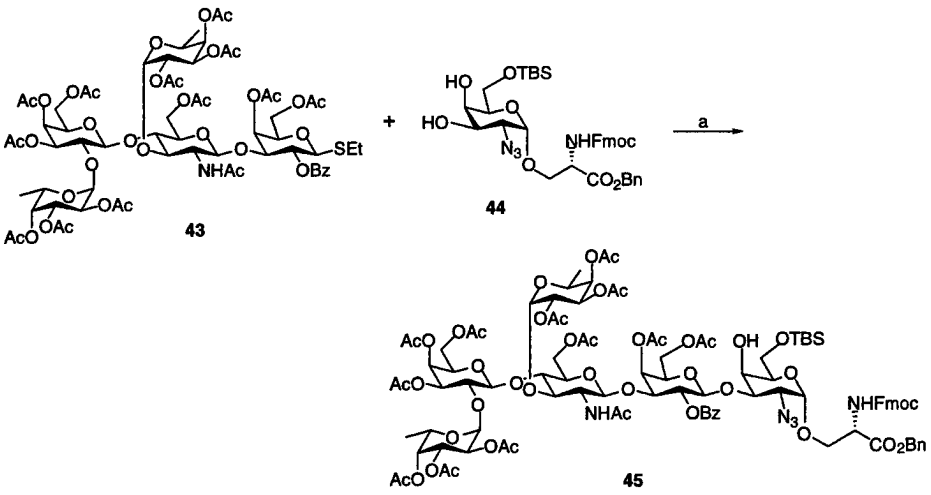
In conclusion, we have successfully prepared the 2,3-STF, STn, 2,6-STF, and glycophorin antigens utilizing both the classical and cassette approaches. Clustered forms of these antigens are now being studied for their immunological profiles. Also, the synthesis of an α-*O*-linked serine glycoside of an entire Lewis blood group (Y) antigen has been accomplished via the cassette methodology. While complexities will undoubtedly be encountered on a case-to-case basis, we believe that the results shown here constitute validation and broad demonstration that the required chemistry can be achieved in the general case. Increasingly sophisticated and, we hope, increasingly realistic cell-surface molecular mimics can be now assembled and evaluated both as regards to spectroscopy and immune recognition.

Experimental Section

Glycal 6. Galactal **5** (1.96 g, 9.89 mmol) was dissolved in 100 mL of anhydrous CH_2Cl_2 and cooled to 0 °C. DMDO (0.06 M solution in acetone, 200 mL, 12 mmol) was added via cannula to the reaction flask. After 1 h the starting material was consumed as judged by TLC. The solvent was removed with a stream of N_2, and the crude epoxide was dried in vacuo for 1 h at ambient temperature. The crude residue was dissolved up in 33 mL of THF, and 6-*O*-TIPS galactal (2.50 g, 8.24 mmol) in 20 mL THF was added. The resulting mixture was cooled to −78 °C, and $ZnCl_2$ (1.0 M solution in ether, 9.80 mL, 9.80 mmol) was added dropwise. The reaction was allowed to warm slowly to ambient temperature and stirred 12 h. The mixture was diluted with EtOAc and washed with $NaHCO_3$ and brine, dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography

(33) Meldal, M.; Bielfeldt, T.; Peters, S.; Jensen, K. J.; H. Paulsen,; Bock, K. *Int. J. Pept. Protein Res.* **1994**, *43*, 529.
(34) Thomas, D. B.; Winzler, R. J. *J. Biol. Chem.* **1969**, *244*, 5943.
(35) Iijima, H.; Ogawa, T. *Carbohydr. Res.* **1989**, *186*, 107.
(36) (a) Nakahara, Y.; Iijima, H.; Ogawa, T. *Tetrahedron Lett.* **1994**, *35*, 3321. (b) Nakahara, Y.; Iijima, H.; Ogawa, T. *Carbohydr. Lett.* **1994**, *1*, 99.
(37) Glunz, P. W.; Hintermann, S.; Lloyd, K. O.; Danishefsky, S. J. Unpublished results.

Scheme 10



(40 → 45 → 50 → 60% EtOAc in hexanes) to yield 3.36 g of product which was immediately acetylated. The material was dissolved in 50 mL of dry CH_2Cl_2, and triethylamine (19.2 mL) and DMAP (20 mg) were added. The solution was cooled to 0 °C at which time acetic anhydride (9.90 mL) was added dropwise. The reaction was stirred at ambient temperature 12 h. The solvent was removed in vacuo and the crude material purified by flash chromatography (50% EtOAc in hexanes) to give 3.30 g (75%) of glycal **6**: ^{1}H NMR ($CDCl_3$) δ 6.42 (d, J = 6.3 Hz, 1H, H-1, glycal), 4.35 (1/2 AB, dd, J = 11.5, 6.8 Hz, 1H, H-6′a), 4.28 (1/2 AB, dd, J = 11.5, 6.1 Hz, 1H, H-6′b). HRMS (EI) calcd for $C_{28}H_{44}O_{13}SiK$ (M + K): 655.2188; found: 655.2154.

Glycal 7. Glycal **6** (1.50 g, 2.43 mmol) was dissolved in 24 mL of THF and cooled to 0 °C. A mixture of TBAF (5.80 mL, 5.83 mmol) and acetic acid (0.34 mL, 5.83 mmol) was added to the substrate at 0 °C. The reaction was stirred at 30 °C for 5 h. The solution was partitioned between EtOAc and saturated $NaHCO_3$, and the phases separated. The organic phase was washed with saturated $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography (80 → 85 → 90% EtOAc/ hexane) to yield 0.9 g (80%) of glycal **7**: ^{1}H NMR ($CDCl_3$) δ 6.38 (dd, J = 6.3, 1.8 Hz, 1H, H-1, glycal), 5.39 (m, 1H, H-4), 2.22 (s, 3H), 2.16 (s, 3H), 2.13 (s, 3H). HRMS (EI) calcd for $C_{19}H_{24}O_{13}K$ (M + K): 499.0854; found: 499.0885.

Glycal 9. A flame-dried flask was charged with sialyl phosphite donor **8** (69 mg, 0.11 mmol) and acceptor **7** (40 mg, 0.085 mmol) in the drybox (argon atmosphere). The mixture was dissolved in 0.6 mL of dry THF. Dry toluene (0.6 mL) was added, and the solution was slowly cooled to −60 °C to avoid precipitation. Trimethylsilyl triflate (2.4 μL) was added, and the mixture was stirred at −45 °C for 2 h. The reaction was quenched at −45 °C with 2 mL of saturated $NaHCO_3$, warmed until the aqueous phase melted, and poured into EtOAc. The organic phase was washed with sat. $NaHCO_3$, dried (Na_2SO_4), and concentrated. ^{1}H NMR analysis of the crude material revealed a 4:1 ratio of α:β isomers. The mixture was separated by flash chromatography on silica gel (2 → 2.5 → 3 → 3.5 → 4% MeOH in CH_2Cl_2) to yield 50 mg (63%) of glycal **9**: ^{1}H NMR ($CDCl_3$) δ 6.42 (d, J = 6.2 Hz, 1H), 5.37 (m, 1H), 5.32−5.29 (m, 4H), 5.26−5.24 (m, 1H), 5.12−5.10 (m, 2H), 4.98 (d, J = 3.5 Hz, 1H), 4.92−4.85 (m, 1H), 4.83−4.80 (m, 3H), 4.54 (m, 1H), 4.45 (dd, J = 3.0, 13.5 Hz, 1H), 4.33−4.20 (m, 3H), 4.22−4.02 (m, 7H), 3.96 (dd, J = 10.9, 7.6 Hz, 1H, H-2), 2.59 (dd, J = 12.9, 4.6 Hz, 1H, H-2eq NeuNAc), 2.30 (dd, J = 12.9, 7.2 Hz, 1H, H-2ax NeuNAc), 2.16 (s, 3H), 2.14 (s, 3H), 2.13 (s, 3H), 2.12 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.88 (s, 3H, CH_3CONH); FTIR (neat) 2959, 1816, 1745, 1684, 1662 cm^{-1}. HRMS (EI) calcd for $C_{39}H_{51}NO_{25}K$ (M + K): 972.2386; found: 972.2407.

Azidonitration Product 10. Glycal **9** (0.37 g, 0.40 mmol) was dissolved in 2.2 mL of dry acetonitrile, and the solution was cooled to −20 °C. Sodium azide (38.6 mg, 0.59 mmol) and ammonium cerium nitrate (0.65 g, 1.20 mmol) were added, and the mixture was vigorously stirred at −15 °C for 12 h. The heterogeneous mixture was diluted with ethyl acetate, washed twice with ice cold water, dried (Na_2SO_4), and concentrated. The residue was purified by flash chromatography to provide 0.25 g (60%) of α:β−anomeric mixture **10**: ^{1}H NMR ($CDCl_3$) δ 6.35 (d, J = 4.2 Hz, 1H, H-1, α-nitrate), 3.79 (s, 3H, methyl ester), 3.41 (dd, J = 11.0, 4.7 Hz, 1H, H-2), 2.54 (dd, J = 4.6, 12.8, H-2eq NeuNAc); ^{13}C NMR ($CDCl_3$, selected characteristic peaks) 170.99, 170.82, 170.30, 170.22, 169.81, 168.58 (acetates), 100.36, 98.34, 97.33, 94.76 (anomeric centers), 72.23, 71.77, 69.29, 68.91, 68.46, 67.70, 67.22, 62.53, 62.50, 57.53, 52.95, 21.06, 20.93, 20.83, 20.78; FTIR (neat): 2117, 1734 cm^{-1}. MS (EI) calcd: 1037.8; found 1038.4 (M + H). The azidonitrate mixture was of limited stability which precluded HRMS analysis.

α-Bromide 11. To a solution of the azidonitrates **10** (0.15 g, 0.15 mmol) in 0.6 mL of dry acetonitrile was added lithium bromide (62.7 mg, 0.73 mmol) and stirred at ambient temperature for 3 h in the dark. The heterogeneous mixture was diluted with dichloromethane, and the solution was washed twice with water, dried ($MgSO_4$), and concentrated (without heating). The residue was purified by flash chromatography (5% MeOH in CH_2Cl_2) to afford 0.12 g (75%) of α-bromide **12** which was stored under an argon atmosphere at −80 °C: ^{1}H NMR ($CDCl_3$) δ 6.54 (d, J = 3.7 Hz, 1H, H-1), 3.40 (dd, J = 10.8, 4.5 Hz, 1H, H-2), 2.57 (dd, J = 12.9, 4.5 Hz, 1H, H-2eq NeuNAc), 2.20 (s, 3H), 2.15 (s, 3H), 2.14 (s, 3H), 2.12 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.87 (s, 3H, CH_3CONH); MS (EI) calcd for $C_{39}H_{51}N_4O_{25}Br$: 1055.7; found: 1057.4 (M + H).

Trichloroacetimidates 12. Azidonitrate **10** (0.60 g, 0.58 mmol) was dissolved in 3.6 mL of acetonitrile, and the resultant solution was treated with thiophenol (0.18 mL) and diisopropylethylamine (0.10 mL). After 10 min, the solvent was removed with a stream of nitrogen. The crude material was purified by flash chromatography (2 → 2.5 → 3 → 3.5% MeOH in CH_2Cl_2) to provide 0.47 g (82%) of intermediate hemiacetal as a 1:1 mixture of α:β-anomers: ^{1}H NMR ($CDCl_3$, selected peaks) δ 5.57 (d, J = 2.9 Hz, 1H, 1α), 5.30−5.35 (m, 4H), 5.21 (d, J = 8.6 Hz, 1H, 1β), 5.11 (m, 4H), 5.04 (dd, J = 3.7, 0.5 Hz, 1H), 4.82−4.91 (m, 4H), 4.79 (dd, J = 8.4, 3.2 Hz, 2H), 4.56 (dd, J = 7.6, 5.9 Hz, 1H), 3.79 (s, 3H), 3.47 (dd, J = 10.4, 4.2 Hz, 1H), 3.34 (dd, J = 10.7, 7.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, selected peaks) δ 100.45, 100.31, 98.98, 98.30, 96.62, 92.19, 23.17, 21.00, 20.85, 20.71; FTIR (neat): 2112, 1747 cm^{-1}. HRMS (EI) calcd for $C_{39}H_{52}N_4O_{26}$ (M + K): 1031.2507; found: 1031.2539. To this hemiacetal (60 mg, 0.06 mmol) in 2.0 mL of CH_2Cl_2 were added trichloroacetonitrile (60 μL) and potassium carbonate (60 mg). After 6 h the mixture was diluted with CH_2Cl_2, filtered of excess K_2CO_3, and concentrated. The residue was purified by flash chromatography (5% MeOH in CH_2Cl_2) to provide 53.2 mg (64%, two steps) of **12** as a 1:1 mixture of α:β-anomers: ^{1}H NMR ($CDCl_3$) δ 8.76 (d, J = 5.1 Hz, 1H), 6.50 (d, J = 3.1 Hz, 1H, α-anomer), 5.65 (d, J = 8.4 Hz, 1H, β-anomer). LRMS (ES) calcd for $C_{41}H_{52}N_5O_{26}Cl_3Na$ (M + Na): 1158.1; found: 1158.2. The low stability

of **12** precluded further characterization. It is recommended to use **12** immediately in the following step.

Compound 14. A flame-dried flask was charged with donor **11** (50 mg, 0.04 mmol), 80 mg of 4 Å mol sieves, and *N*-Fmoc-L-serine benzyl ester (27.5 mg, 0.07 mmol) in the drybox. THF (0.6 mL) was added to the flask, and the mixture was cooled to −30 °C. Boron trifluoride diethyl etherate (2.8 μL, 0.022 mmol) was added, and the reaction was allowed to warm to −10 °C over 3 h. The mixture was diluted with EtOAc and washed with sat. $NaHCO_3$ while still cold. The organic phase was dried (Na_2SO_4) and concentrated, and the residue was purified by flash chromatography (2 → 2.5 → 3% MeOH in CH_2Cl_2) to provide 40 mg (66%) of **14** as a 4:1 mixture of α: β-isomers. The pure α-anomer was separated by flash chromatography (80 → 85 → 90 → 100% EtOAc:hexanes). **14α**: $[\alpha]^{23}_D$ +34.3 (*c* 0.44, $CDCl_3$); ^1H NMR ($CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.62 (br d, 2H), 7.30−7.48 (m, 9H), 6.09 (br d, *J* = 7.9 Hz, 1H), 5.70 (d, *J* = 8.8 Hz, 1H), 5.34−5.39 (m, 2H), 5.30 (m, 1H), 5.20−5.27 (m, 1H), 5.17 (br t, *J* = 9.9 Hz, 2H), 4.79−4.95 (m, 5H), 4.80 (dd, *J* = 3.1 Hz, 1H), 4.60 (br s, 1H), 4.38−4.48 (m, 4H), 4.22−4.31 (m, 3H), 3.99−4.11 (m, 5H), 3.82−3.95 (m, 3H), 3.75 (s, 3H), 3.33 (dd, *J* = 10.2, 4.4 Hz, 1H), 2.55 (dd, *J* = 12.8, 4.5 Hz, 1H, H_{2eq} of NeuNAc), 2.11 (s, 3H), 2.10 (s, 3H), 2.08 (s, 6H), 2.07 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.99 (s, 3H), 1.93 (app t, *J* = 12.5 Hz, 1H, H_{2ax} of NeuNAc); ^{13}C NMR ($CDCl_3$) δ 170.92, 170.73, 170.60, 170.22, 170.19, 170.11, 169.81, 168.96, 167.81, 152.68, 143.79, 141.34, 134.90, 128.82, 128.70, 128.41, 127.85, 127.15, 125.12, 120.06, 100.43, 99.08, 98.66, 75.80, 73.04, 72.69, 72.25, 69.23, 69.09, 68.73, 68.49, 67.59, 67.40, 67.24, 63.78, 62.48, 62.24, 54.71, 52.91, 49.35, 48.87, 47.18, 37.64, 23.30, 23.22, 21.00, 20.84, 20.79, 20.76; IR (neat): 1746, 1673 cm^{-1}. HRMS: Calcd for $C_{66}H_{77}N_3O_{31}K$: 1446.4178; found: 1446.4140.

Compound 15. A flame-dried flask was charged with silver perchlorate (27.3 mg, 0.14 mmol), 0.12 g of 4 Å mol sieves, and *N*-Fmoc-L-threonine benzyl ester (37.3 mg, 0.086 mmol) in the drybox. Dry CH_2Cl_2 (0.72 mL) was added to the flask, and the mixture was stirred at ambient temperature for 10 min. Bromide **11** (76 mg, 0.072 mmol) in 0.46 mL of CH_2Cl_2 was added slowly over 40 min. The reaction was stirred under argon atmosphere at ambient temperature for 2 h. The mixture was then diluted with CH_2Cl_2 and filtered through Celite. The precipitate was thoroughly washed with CH_2Cl_2, the filtrate was evaporated, and the crude material was purified by flash chromatography (1 → 1.5 → 2 → 2.5% MeOH in CH_2Cl_2) to provide 74 mg (74%) of **15**. The undesired β-anomer was not detected by ^1H NMR and HPLC analysis of the crude material. **15**: $[\alpha]^{23}_D$ +26.8 (*c* 0.45, CH_2Cl_2); ^1H NMR ($CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.63 (d, *J* = 7.2 Hz, 2H), 7.40−7.25 (m, 8H), 5.72 (d, *J* = 9.2 Hz, 1H), 5.46 (s, 1H), 5.33 (m, 1H), 5.29 (d, *J* = 8.2 Hz, 1H), 5.23 (s, 2H), 5.11−5.04 (m, 3H), 4.87−4.71 (m, 4H), 4.4−4.39 (m, 3H), 4.33−4.25 (m, 4H), 4.09−3.97 (m, 6H), 3.79 (s, 3H), 3.66 (dd, *J* = 10.6, 3.7 Hz, 1H, H-3), 3.38 (dd, *J* = 10.7, 3.0 Hz, 1H, H-2), 2.52 (dd, *J* = 12.7, 4.3 Hz, 1H, H-2eq NeuNAc), 2.20 (s, 3H), 2.13 (s, 3H), 2.11 (s, 3H), 2.10 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.87 (s, 3H, *CH*3CONH), 1.35 (d, *J* = 6.2 Hz, Thr-CH_3); FTIR (neat) 2110, 1749; HRMS (EI) calcd for $C_{65}H_{75}N_5O_{30}K$ (M + K): 1444.4130; found: 1444.4155.

Protected 2,6-STF Threonine Acid 17. Azide **15** (47 mg, 33.4 μmol) was treated with thiolacetic acid (3 mL, distilled three times) for 27 h at ambient temperature. Excess thiolacetic acid was removed with a stream of nitrogen, followed by toluene azeotrope (4 × 5 mL). The crude product was purified by flash chromatography (1.5 → 2 → 2.5 → 3 → 3.5% MeOH in CH_2Cl_2) to yield 37 mg (78%) of reduced product which was immediately dissolved in 7.6 mL of methanol and 0.5 mL of water. After purging the system with argon, 6.5 mg of palladium catalyst (10% Pd−C) was added and the system placed under 1 atm of H_2. After 8 h the H_2 was removed by an argon flow, the catalyst was removed by filtration, and the mixture was concentrated. Flash chromatography of the residue (10% MeOH in CH_2Cl_2) provided 36 mg (78%) of acid 17: $[\alpha]^{23}_D$ +34.7 (*c* 1.75, CDCl3); ^1H NMR ($CDCl_3$) mixture of rotamers, selected characteristic peaks: δ 3.80 (s, 3H, methyl ester), 3.41 (m, 1H, H-2), 2.53 (m, 1H, H-2e NeuNAc)), 1.45 (d, *J* = 5.1 Hz, Thr-CH_3), 1.35 (d, *J* = 5.8 Hz, Thr-CH_3); FTIR (neat) 1818, 1747; HRMS (EI) calcd for $C_{60}H_{73}N_3O_{31}K$ (M + K): 1370.3870; found: 1370.3911.

Protected 2,6-STF Serine Acid 16. The compound **16** was prepared in 80% yield from **14** following the same procedure for the preparation of **17**: $[\alpha]^{23}_D$ +40.0 (*c* 1.75, $CDCl_3$); ^1H NMR ($CDCl_3$) mixture of rotamers, selected characteristic peaks: δ 3.36 (br s), 3.28 (br s), 2.52 (br d), 2.48 (br d); ^{13}C NMR ($CDCl_3$) selected peaks: δ 98.90, 98.60, 77.04, 76.79, 72.61, 69.10, 68.56, 68.43, 67.56, 62.68, 62.42, 53.09, 49.33, 47.16, 23.18, 21.02, 20.84, 20.75, 20.66, 20.22; FTIR (KBr pellet): 3362, 1750 cm^{-1}. HRMS (EI) calcd for $C_{59}H_{71}N_3O_{31}K$ (M + K): 1356.3708; found: 1356.3820.

General Procedure for Peptide Coupling. Glycosyl amino acid **16** or **17** (1 equiv) and the peptide with a free amino group (1.2 equiv) were dissolved in CH_2Cl_2 (22 mL/1 mmol). The solution was cooled to 0 °C, and IIDQ (1.15−1.3 equiv) was added (1 mg in ca. 0.02 mL of CH_2Cl_2). The reaction was then stirred at ambient temperature for 8 h. The mixture was directly loaded onto a silica gel column for purification by flash chromatography.

General Procedure for *N*-Fmoc Deprotection. A substrate (1 mmol in 36 mL of DMF) was dissolved in anhydrous DMF followed by addition of KF (10 equiv) and 18-crown-6 ether (catalytic amount). The mixture was then stirred for 48 h at ambient temperature. Evaporation of DMF in vacuo was followed by purification by flash chromatography on silica gel.

Protected Glycopeptide 18. ^1H NMR ($CDCl_3$) δ 3.45−3.30 (m, 3 × 1H, H-2), 3.74 (s, 3H, methyl ester), 2.58−2.49 (m, 3 × 1H, H-2eq NeuNAc); FTIR (neat) 2962, 1819, 1747, 1664; MS (EI) calcd: 3760; found: 1903.8/doubly charged = 3806 (M + 2Na).

Deprotected Glycopeptide 19. The benzyl ester was hydrogenolyzed according to the procedure set forth for compound **17**. The acid was dissolved in methanol to yield a 5 μM solution. 1 M NaOH (aq) was added dropwise until the pH reached 10−10.5, and the mixture was stirred for 12 h at ambient temperature. Amberlyst-18 was added to bring the pH to ca. 4. The resin was filtered off and the mixture concentrated. The crude product was purified by reverse phase flash chromatography (LiChroprep RP-18, H_2O eluant) to provide fully deprotected **19** in 80% yield (two steps): ^1H NMR (D_2O) δ 4.73 (m, 2H, 2 × H-1), 4.70 (d, 1H, H-1), 4.64 (m, 3H, 3 × H-1′), 4.26−4.20 (m, 5H), 4.12−4.00 (m, 7H), 3.95−3.82 (m, 7H), 3.77−3.27 (m, 51H), 2.55−2.51 (m, 3H, 3 × H-2eq NeuNAc), 1.84−1.82 (m, 21H, CH_3-CONH), 1.52−1.45 (m, 3H, H-2ax NeuNAc), 1.20 (d, *J* = 7.2 Hz, 3H), 1.18 (d, *J* = 6.6 Hz, 3H), 1.12 (d, *J* = 6.2 Hz, 3H), 0.71 (d, *J* = 6.6 Hz, 6H, Val); ^{13}C NMR (D_2O) anomeric carbons: δ 105.06, 105.01, 100.60, 100.57, 100.53, 100.11, 99.52, 98.70; MS (FAB) $C_{96}H_{157}N_{11}O_{64}$ 2489 (M + H); MS(MALDI) 2497.

Compound 25. To a suspension of 6-acceptor **21** (R = Me, 0.41 g, 0.62 mmol), sialyl phosphite **22** (X = $OP(OBn)_2$, 0.42 g, 0.57 mmol), and 0.2 g 4 Å mol sieves in 15 mL of dry THF at −45 °C was added trimethylsilyl trifluoromethanesulfonate (23 μL, 0.11 mmol) dropwise over 2 min. The mixture was stirred at −45 °C for 12 h at which time an additional 23 μL of TMSOTf was added dropwise. The mixture was stirred 6 h at −45 °C, and then filtered through a pad of Celite and concentrated. Flash chromatography of the residue (1:1 hexanes: EtOAc → EtOAc) yielded a mixture of α- and β-anomers, which was subjected to further chromatography (4:1 EtOAc:hexanes) to afford 0.26 g (37%) of pure α anomer **25** (R = Me) as a colorless foam. $[\alpha]^{23}_D$ +32.6 (*c* 1.18, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.20−7.37 (m, 10H), 5.65 (d, *J* = 9.5 Hz, 1H), 5.41 (d, *J* = 9.3 Hz, 1H), 5.36 (m, 1H), 5.30 (d, *J* = 7.5 Hz, 1H), 5.18 (dd, *J* = 12.1, 5.8 Hz, 2H), 4.85 (m, 1H), 4.76 (d, *J* = 3.6 Hz, 1H), 4.41 (m, 3H), 4.32 (dd, *J* = 12.4, 2.6 Hz, 1H), 4.27 (m, 2H), 4.20 (app t, *J* = 7.3 Hz, 1H), 4.04−4.12 (m, 5H), 3.87 (m, 1H), 3.74 (s, 3H), 3.63 (m, 1H), 3.29 (dd, *J* = 7.6, 3.6 Hz, 1H), 2.56 (dd, *J* = 12.9, 4.7 Hz, 1H), 2.09 (s, 3H), 2.08 (s, 3H), 2.02 (m, 1H), 1.98 (s, 6H), 1.84 (s, 3H), 1.45 (s, 3H), 1.32 (d, *J* = 5.5 Hz, 3H), 1.31 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 170.81, 170.44, 170.14, 170.00, 169.94, 169.90, 156.63, 143.84, 143.57, 141.18, 141.15, 134.93, 128.56, 128.51, 128.47, 127.62, 127.59, 127.01, 126.97, 125.14, 125.06, 119.85, 109.82, 98.84, 98.59, 77.20, 72.97, 72.70, 72.37, 69.07, 68.98, 67.55, 67.48, 67.24, 67.17, 63.50, 62.26, 60.75, 58.86, 52.65, 49.22, 47.03, 37.35, 27.87, 25.97, 23.04, 20.95, 20.72, 20.68, 20.63, 18.56; IR (neat): 2986, 2109, 1745, 1668, 1666 cm^{-1}.

Protected STn Acid 26. $[\alpha]^{23}_D$ +36.7 (*c* 1.04, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.83 (d, *J* = 7.5 Hz, 2H), 7.70 (app t, *J* = 6.7 Hz, 2H), 7.41 (m, 2H), 7.32 (m, 2H), 5.41 (m, 2H), 5.33 (dd, *J* = 9.2, 2.0 Hz, 1H), 5.03 (dd, *J* = 11.7, 3.2 Hz, 1H), 4.93 (d, *J* = 3.8 Hz, 1H), 4.81 (m, 1H), 4.64 (dd, *J* = 10.8, 6.3 Hz, 1H), 4.46 (dd, *J* = 10.7, 5.9 Hz, 1H), 4.37 (m, 2H), 4.27 (m, 3H), 4.14 (m, 2H), 4.08 (dd, *J* = 12.4, 5.1 Hz, 1H), 3.97 (app t, *J* = 10.5 Hz, 1H), 3.87 (m, 1H), 3.82 (s, 3H), 3.32 (m, 1H), 2.60 (dd, *J* = 12.7, 4.6 Hz, 1H), 2.16 (s, 3H), 2.11 (s, 3H), 2.09 (s, 3H), 2.03 (m, 1H), 2.00 (s, 3H), 1.98 (s, 3H), 1.94 (s, 3H), 1.93 (s, 3H), 1.83 (s, 3H), 1.23 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 175.61, 175.47, 175.34, 174.19, 174.08, 173.83, 173.63, 173.44, 173.30, 171.07, 160.91, 147.30, 147.00, 144.61, 144.58, 130.70, 130.65, 130.11, 130.05, 128.04, 127.87, 122.90, 122.83, 102.71, 101.75, 79.98, 75.16, 72.49, 71.90, 71.12, 70.89, 70.38, 69.38, 66.45, 65.33, 61.94, 55.29, 51.89, 40.74, 24.75, 24.56, 23.08, 22.78, 22.62, 22.57, 21.20; IR (neat): 3361, 2956, 1746, 1659 cm^{-1}. HRMS: Calcd for $C_{51}H_{63}N_3O_{24}$-Na: 1124.3699; found: 1124.3748.

Protected STn Cluster 27. $[\alpha]^{23}_D$ +42.0 (*c* 1.67, $CHCl_3$); ^1H NMR (CD_3OD) δ 7.83 (d, *J* = 7.5 Hz, 2H), 7.72 (app t, *J* = 6.5 Hz, 2H), 7.41 (app t, *J* = 7.5 Hz, 2H), 7.34 (app t, *J* = 7.4 Hz, 2H), 5.34 (m, 8H), 5.17 (m, 2H), 5.08 (m, 2H), 4.98 (d, *J* = 3.6 Hz, 1H), 4.90 (d, *J* = 2.7 Hz, 1H), 4.78 (m, 2H), 4.69 (br s, 1H), 4.58 (dd, *J* = 10.7, 6.6 Hz, 1H), 4.49 (dd, *J* = 10.6, 6.2 Hz, 1H), 4.42 (m, 3H), 4.27−4.35 (m, 8H), 4.18 (app t, *J* = 5.9 Hz, 1H), 4.09 (m, 6H), 3.96 (m, 2H), 3.88 (m, 2H), 3.81 (s, 3H), 3.71 (s, 3H), 3.08 (m, 3H), 2.59 (dd, *J* = 12.7, 4.6 Hz, 2H), 2.54 (dd, *J* = 12.8, 4.6 Hz, 1H), 2.17 (s, 3H), 2.16 (s, 3H), 2.11 (s, 9H), 2.09 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.98 (s, 6H), 1.97 (s, 3H), 1.97 (s, 3H), 1.94 (s, 3H), 1.93 (s, 3H), 1.92 (s, 3H), 1.83 (s, 3H), 1.82 (s, 6H), 1.45 (s, 9H), 1.37 (m, 6H), 1.32 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (CD_3OD) δ 173.78, 173.53, 173.25, 172.70, 172.40, 172.36, 172.31, 172.23, 172.06, 171.82, 171.79, 171.77, 171.54, 171.50, 169.24, 169.19, 169.14, 159.12, 158.56, 145.49, 145.22, 142.76, 128.91, 128.29, 126.32, 126.20, 121.11, 121.05, 101.24, 100.63, 100.01, 99.98, 99.95, 80.06, 79.41, 78.35, 73.34, 71.02, 70.71, 70.40, 69.24, 68.55, 67.79, 64.76, 63.44, 60.32, 58.53, 58.24, 53.48, 50.06, 38.95, 38.08, 30.91, 28.94, 23.55, 23.44, 23.39, 22.74, 21.30, 21.04, 20.87, 20.79, 19.87, 19.62; IR (neat): 2934, 2470, 1746, 1654 cm^{-1}.

Thioglycoside Donor 33. To a solution of lactone **30** (0.19 g, 0.26 mmol) in 8.5 mL of CH_2Cl_2 at 0 °C was added freshly prepared DMDO (0.06 M solution in acetone, 8.5 mL, 0.51 mmol). The solution was stirred for 1 h, and the solvent was evaporated with an N_2 flow. To the residue was added 3.0 mL of CH_2Cl_2, and the solution was cooled to −78 °C. Ethanethiol (0.38 mL, 5.11 mmol) was added, followed by trifluoroacetic anhydride (2 μL, 0.01 mmol). The mixture was allowed to warm slowly to ambient temperature over 6 h, and concentrated with an N_2 flow. Flash chromatography of the residue (4:1, EtOAc: hexanes) yielded 0.13 g (61%) of thioglycoside **33** as colorless crystals: $[\alpha]^{23}_D$ −22.8 (*c* 4.2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 5.50 (ddd, *J* = 7.6, 2.0 Hz, 1H), 5.41 (d, *J* = 10.2 Hz, 1H), 5.23 (dd, *J* = 8.2, 1.6 Hz, 1H), 5.18 (ddd, *J* = 6.2, 2.2 Hz, 1H), 4.83 (d, *J* = 4.0 Hz, 1H), 4.36−4.31 (m, 2H), 4.23−4.08 (m, 3H), 4.00−3.88 (m, 2H), 3.72 (d, *J* = 7.1 Hz, 1H), 3.65 (dd, *J* = 10.3, 1.4 Hz, 1H), 3.51 (t, *J* = 9.4 Hz, 1H), 2.81−2.57 (m, 3H), 2.46 (dd, *J* = 13.5, 5.4 Hz, 1H), 2.14 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.01 (m, 1H), 2.01 (s, 3H), 2.00 (s, 3H), 1.87 (s, 3H), 1.30 (t, *J* = 7.3 Hz, 3H), 1.13 (bs, 21H); ^{13}C NMR ($CDCl_3$) δ 171.0, 170.6, 170.5, 170.4, 170.0, 169.8, 164.0, 95.0, 85.1, 78.6, 77.2, 74.5, 73.2, 69.7, 69.5, 68.7, 66.9, 62.7, 62.2, 49.1, 38.5, 24.3, 23.0, 20.8, 20.6, 20.5, 17.8, 15.1, 11.8; IR (film) 3364, 2942, 1749, 1667 cm^{-1}; HRMS: Calcd for $C_{36}H_{59}NO_{16}SSiNa$: 844.3222; found: 844.3227.

Benzoate Thioglycoside Donor 35. To a solution of thio-donor **33** (0.21 g, 0.26 mmol) in 4.0 mL of CH_2Cl_2 were added triethylamine (0.18 mL, 1.28 mmol), benzoyl chloride (0.15 mL, 1.28 mmol), and DMAP (0.31 g, 2.55 mmol), and the solution was stirred at ambient temperature for 16 h. The mixture was partitioned between EtOAc (50 mL) and sat. $NaHCO_3$ (50 mL), and the phases were separated. The organic phase was washed with brine (50 mL), dried (Na_2SO_4), and concentrated. To the crude benzoate **34** in 10 mL of THF at 0 °C were added acetic acid (0.89 mL, 15.6 mmol) and TBAF (1.0 M solution in THF, 3.90 mL, 3.90 mmol). The solution was allowed to warm slowly to ambient temperature and stirred for 16 h. The solution was partitioned between EtOAc (60 mL) and H_2O (60 mL), and the phases were separated. The organic phase was washed with sat. $NaHCO_3$ (60 mL) and brine (60 mL), dried (Na_2SO_4), and concentrated. To the crude alcohol was added triethylamine (0.72 mmol, 5.20 mmol), acetic anhydride (0.49 mL, 5.20 mmol), and DMAP (10 mg, 0.08 mmol), and the solution was stirred for 6 h at ambient temperature. The mixture was partitioned between EtOAc (50 mL) and sat. $NaHCO_3$ (50 mL), and the phases were separated. The organic phase was washed with brine (50 mL), dried (Na_2SO_4), and concentrated. Flash chromatography of the residue (4:1, EtOAc:hexanes) provided 0.15 g (71% from **33**) of thioethyl glycoside **35** as a pale yellow solid. $[\alpha]^{23}_D$ +1.56 (*c* 1.58, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.00 (d, *J* = 7.2 Hz, 2H), 7.58 (t, *J* = 7.4 Hz, 1H), 7.44 (t, *J* = 7.6 Hz, 2H), 5.50 (ddd, *J* = 16.1, 11.0, 5.4 Hz, 1H), 5.40 (m, 1H), 5.24 (m, 3H), 5.08 (d, *J* = 3.6 Hz, 1H), 4.63 (dd, *J* = 12.0, 3.1 Hz, 1H), 4.55 (d, *J* = 10.0 Hz, 1H), 4.34 (m, 3H), 4.10 (ABq, *J* = 10.4 Hz, 1H), 3.94 (m, 2H), 3.65 (d, *J* = 10.5 Hz, 1H), 2.68 (m, 1H), 2.48 (dd, *J* = 13.8, 5.4 Hz, 1H), 2.10 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H), 1.97 (s, 3H), 1.85 (s, 3H), 1.77 (dd, *J* = 13.7, 11.7 Hz, 1H), 1.22 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 170.82, 170.65, 170.61, 170.39, 170.30, 169.90, 165.16, 163.14, 133.39, 129.77, 129.14, 128.39, 95.09, 82.87, 74.59, 72.71, 72.62, 69.23, 68.87, 68.42, 66.93, 62.79, 62.18, 48.99, 38.04, 24.07, 23.03, 20.77, 20.70, 20.66, 20.55, 14.84; IR (neat): 2966, 1746, 1676 cm^{-1}. HRMS: Calcd for $C_{36}H_{45}NO_{18}SNa$: 834.2255; found: 834.2269.

Compound 37. A mixture of thiodonor **35** (0.10 g, 0.12 mmol) and 3-acceptor **32** (0.18 g, 0.25 mmol) was azeotroped with dry benzene (4 × 5 mL), and the flask was backfilled with nitrogen and placed under high vacuum for 1 h. Molecular sieves (4 Å, 0.5 g), CH_2Cl_2 (5.0 mL), and NIS (69 mg, 0.31 mmol) were added, and the mixture was cooled to 0 °C. Triflic acid (freshly prepared 1% solution in CH_2Cl_2, 1.84 mL, 0.12 mmol) was added dropwise over 5 min. The mixture was warmed to ambient temperature immediately following addition, allowed to stir for 10 min, and then partitioned between EtOAc (50 mL) and sat. $NaHCO_3$ (50 mL). The phases were separated, and the aqueous phase was back-extracted with EtOAc (20 mL). The combined organic phases were washed with sat. $Na_2S_2O_3$ (50 mL) and brine (50 mL), dried (Na_2SO_4), and concentrated. Flash chromatography of the residue (4:1 EtOAc:hexanes) furnished 0.11 g (62%) of trisaccharide **37** as a colorless crystalline solid. $[\alpha]^{23}_D$ +29.6 (*c* 1.65, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.02 (d, *J* = 7.3 Hz, 2H), 7.77 (d, *J* = 7.7 Hz, 2H), 7.56 (m, 2H), 7.26−7.50 (m, 12H), 5.59 (d, *J* = 9.5 Hz, 1H), 5.51 (ddd, *J* = 15.9, 11.2, 5.5 Hz, 1H), 5.59 (d, *J* = 9.5 Hz, 1H), 5.21 (br s, 4H), 5.07 (m, 3H), 4.85 (d, *J* = 8.0 Hz, 1H), 4.66 (m, 2H), 4.19−4.48 (m, 10H), 4.13 (br s, 1H), 4.66 (m, 2H), 4.19−4.48 (m, 10H), 4.13 (br s, 1H), 4.09 (d, *J* = 10.4 Hz, 1H), 4.04 (m, 1H), 3.94 (m, 3H), 3.78 (m, 4H), 3.64 (d, *J* = 10.4 Hz, 1H), 3.45 (dd, *J* = 10.5, 3.9 Hz, 1H), 2.11 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H), 2.00 (s,3H), 1.99 (s, 3H), 1.86 (s, 3H), 1.78 (m, 1H), 1.29 (d, *J* = 6.3 Hz, 3H), 0.86 (s, 9H) 0.03 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 170.95, 170.66, 170.39, 169.95, 165.30, 163.02, 156.70, 143.92, 143.63, 141.24, 134.81, 133.41, 129.74, 129.11, 128.58, 128.54, 128.49, 128.36, 128.01, 127.71, 127.09, 127.02, 125.17, 125.11, 119.96, 100.80, 99.49, 95.16, 78.46, 76.17, 72.78, 72.14, 71.75, 71.54, 71.25, 70.92, 70.05, 69.18, 68.57, 68.33, 67.61, 67.33, 67.07, 63.05, 62.25, 62.21, 58.79, 58.70, 49.23, 47.11, 37.97, 25.83, 23.10, 20.82, 20.73, 20.71, 20.63, 20.55, 18.78, 18.28, 18.00, 17.88, 17.84, 11.89, −5.35, −5.50; IR (neat): 2953, 2931, 2111, 1744, 1689 cm^{-1}. HRMS: Calcd for $C_{72}H_{87}N_5O_{27}SiNa$: 1504.5255; found: 1504.5202.

Peracetylated 2,3-STF Trisaccharide 39. To trisaccharide **37** (70 mg, 0.05 mmol) in 1.0 mL of THF was added 1.0 mL of 1 N HCl. The solution was stirred for 30 min and then partitioned between EtOAc (30 mL) and sat $NaHCO_3$ (30 mL). The phases were separated, and the organic phase was washed with brine (30 mL), dried (Na_2SO_4), and concentrated. To the residue were added CH_2Cl_2 (2.0 mL), acetic anhydride (0.1 mL), triethylamine (0.1 mL), and DMAP (2 mg), and the solution was stirred at ambient temperature for 12 h. The mixture was partitioned between EtOAc (30 mL) and sat. $NaHCO_3$ (30 mL). The phases were separated, and the organic phase was washed with brine (30 mL), dried (Na_2SO_4), and concentrated. The residue was purified by flash chromatography (4:1 hexanes:EtOAc) to afford 58 mg (85%) of peracetylated trisaccharide **39** as a colorless crystalline

solid. $[\alpha]^{23}_D$ +40.0 (*c* 0.90, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.02 (d, J = 7.4 Hz, 2H), 7.78 (d, J = 7.4 Hz, 2H), 7.59 (d, J = 7.4 Hz, 2H), 7.37–7.53 (m, 6H), 7.29 (m, 6H), 5.61 (d, J = 9.4 Hz, 1H), 5.51 (m, 2H), 5.19 (m, 4H), 5.08 (d, J = 4.3 Hz, 1H), 5.00 (d, J = 3.5 Hz, 1H), 4.81 (d, J = 7.9 Hz, 1H), 4.75 (d, J = 3.8 Hz, 1H), 4.58 (dd, J = 11.9, 3.3 Hz, 1H), 4.47 (dd, J = 10.3, 7.1 Hz, 1H), 4.27–4.42 (m, 5H), 4.17–4.25 (m, 2H), 4.10 (m, 2H), 3.90–4.02 (m, 3H), 3.86 (dd, J = 11.8, 8.2 Hz, 1H), 3.63 (d, J = 10.5 Hz, 1H), 3.46 (dd, J = 10.6, 3.7 Hz, 1H), 2.49 (dd, J = 13.8, 5.5 Hz, 1H), 2.11 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 2.09 (s, 3H), 2.00 (s, 6H), 1.98 (s, 3H), 1.85 (s, 3H), 1.75 (app t, J = 13.7 Hz, 1H), 1.26 (d, J = 6.3 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 170.89, 170.65, 170.36, 169.99, 169.82, 169.50, 165.25, 162.92, 156.70, 143.83, 143.61, 141.27, 134.78, 133.40, 129.72, 129.19, 128.63, 128.59, 128.45, 128.37, 127.76, 127.09, 127.04, 125.11, 125.06, 120.01, 100.95, 99.22, 95.08, 75.29, 72.51, 72.26, 71.74, 71.19, 70.20, 69.54, 69.20, 68.21, 68.05, 67.67, 67.36, 66.78, 63.18, 62.38, 62.22, 59.34, 58.72, 49.28, 47.10, 38.03, 23.11, 20.82, 20.75, 20.71, 20.65, 20.56, 18.38; IR (neat): 3356, 2961, 2111, 1744 cm^{-1}. HRMS: Calcd for $C_{70}H_{77}N_5O_{29}$-Na: 1474.4602; found: 1474.4595.

Azide Reduction of 39. Trisaccharide **39** (77 mg, 0.053 mmol) was deprotected according to the procedure set forth for azide reduction of **17** with AcSH. Purification by flash chromatography (1:1 hexanes: EtOAc → EtOAc) afforded 44 mg (56%) of a colorless oil. $[\alpha]^{23}_D$ +43.2 (*c* 1.65, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.00 (d, J = 7.5 Hz, 2H), 7.79 (d, J = 7.5 Hz, 2H), 7.62 (m, 2H), 7.25–7.53 (m, 12 H), 5.65 (d, J = 9.4 Hz, 1H), 5.45 (m, 1H), 5.41 (br s, 2H), 5.27 (d, J = 10.3 Hz, 1H), 5.15 (m, 2H), 5.01 (d, J = 3.3 Hz, 1H), 4.90 (d, J = 11.9 Hz, 1H), 4.76 (d, J = 3.4 Hz, 1H), 4.72 (d, J = 7.7 Hz, 1H), 4.63 (m, 1H), 4.52 (m, 2H), 4.25–4.43 (m, 6H), 4.18 (m, 2H), 4.08 (m, 2H), 3.93 (m, 2H), 3.84 (dd, J = 11.5, 8.2 Hz, 1H), 3.64 (d, J = 10.5 Hz, 1H), 2.50 (dd, J = 13.5, 5.0 Hz, 1H), 2.11 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 2.01 (s, 3H), 1.99 (s, 6H), 1.95 (s, 3H), 1.81 (s, 3H), 1.73 (app t, J = 12.7 Hz, 1H), 1.23 (s, 3H), 1.22 (d, J = 5.8 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 171.13, 170.02, 170.66, 170.41, 170.37, 169.96, 169.77, 164.89, 162.95, 156.58, 143.72, 141.37, 141.32, 134.41, 133.62, 129.99, 129.81, 129.15, 128.83, 128.76, 128.55, 128.49, 127.80, 127.13, 127.08, 124.93, 124.87, 120.08, 99.85, 99.61, 95.11, 74.15, 72.53, 72.33, 71.57, 71.40, 70.25, 69.15, 68.74, 68.25, 67.95, 67.60, 66.94, 66.78, 63.25, 62.67, 62.21, 58.64, 49.28, 48.71, 47.20, 37.97, 29.65, 23.08, 22.64, 20.80, 20.74, 20.72, 20.65, 20.56, 18.25; IR (neat): 3333, 2927, 1745, 1670 cm^{-1}. HRMS: Calcd for $C_{72}H_{81}N_3O_{30}Na$: 1490.4802; found: 1490.4814. To this oil (29 mg, 0.020 mmol) was added morpholine (2 mL), and the mixture was stirred at ambient temperature 1 h. Excess morpholine was removed by azeotroping with toluene (3 × 5 mL). To the crude free amine in 2.0 mL of CH_2Cl_2 were added acetic anhydride (0.1 mL) and diisopropylethylamine (0.1 mL), and the solution was stirred at ambient temperature 1 h. The mixture was partitioned between EtOAc (20 mL) and saturated $NaHCO_3$ (20 mL). The phases were separated, and the organic phase was dried (Na_2SO_4) and concentrated. Flash chromatography of the residue (3 → 4 → 5 → 6 → 7 → 8% MeOH in CH_2Cl_2) provided 22.8 mg of a colorless solid. The product contained ca. 10% of a byproduct, presumably owing to some lactone opening by morpholine (supported by mass spectral analysis). The mixture was used in the next step without purification. Hydrogenolysis of the benzyl ester according to the procedure set forth for compound **17** was effected and the product used without purification. To the crude acid was added methanol (0.5 mL), 0.1 N aq NaOH (0.5 mL), and the mixture was stirred at ambient temperature 24 h. DOWEX-50H was added to lower the pH to ca. 4, the mixture was filtered through a cotton plug to remove the resin, and the solution was concentrated. To the residue was added methanol (2.0 mL), and then sodium methoxide (25 wt % in MeOH) dropwise until the pH reached 10–10.5 (ca. 5 drops). The solution was heated to reflux 16 h and cooled, and DOWEX-50H was added to lower the pH of the solution to ca. 4. The mixture was filtered through a cotton plug to remove the resin and concentrated. Purification of the residue by reverse-phase column chromatography (LiChroprep RP-18, H_2O eluant) provided 7.1 mg (44%) of *N*-acetyl-2,3-STF **40** as a colorless crystalline solid. $[\alpha]^{23}_D$ +56.1 (*c* 0.27, MeOH); ^1H NMR (D_2O) δ 4.95 (d, J = 3.8 Hz, 1H), 4.53 (d, J = 7.8 Hz, 1H), 4.36 (dd, J = 6.6, 2.6 Hz, 1H), 4.26 (m, 2H), 4.20 (br d, J = 2.6 Hz, 1H), 4.05 (m, 3H), 3.92 (d, J = 3.2 Hz, 1H), 3.86 (m, 4H), 3.72 (m, 4H), 3.62 (m, 4H), 3.51 (dd, J = 9.6, 8.0 Hz, 1H), 2.75 (dd, J = 12.4, 4.6 Hz, 1H), 2.12 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.78 (t, J = 12.2 Hz, 1H), 1.24 (d, J = 6.4 Hz, 3H); ^{13}C NMR (D_2O, external CS_2 reference) δ 176.14, 174.97, 174.61, 174.10, 173.98, 104.57, 99.63, 99.00, 77.23, 76.74, 75.62, 74.78, 72.78, 71.91, 70.95, 69.00, 68.80, 68.45, 68.12, 67.37, 62.53, 61.28, 61.02, 59.26, 51.66, 48.57, 39.77, 22.39, 22.05, 21.97, 18.26; HRMS: Calcd for $C_{31}H_{49}N_3O_{22}Na_3$: 884.2501; found: 884.2551.

Disaccharide Acceptor 41. 6-Acceptor **20** (190 mg, 0.29 mmol), $CaSO_4$ (400 mg), and AgOTf (125 mg, 0.58 mmol) were combined in a flask under argon, at which time 5.0 mL of THF and DTBP (0.15 mL, 0.58 mmol) were added. After stirring at ambient temperature for 30 min, the reaction was cooled to −78 °C, and chloride donor **23** (X = Cl, 0.30 g, 0.58 mmol) in 5.0 mL of THF was added dropwise over 1 h. The reaction was allowed to warm to −10 °C and held at this temperature 4 h and then filtered through Celite and concentrated. The crude product **24** was treated with 80% HOAc for 16 h and concentrated. The resultant oil was purified by silica gel chromatography (2 → 3 → 4% MeOH in CH_2Cl_2) to provide 0.15 g of **41** (48% from **20**) as a white foam: $[\alpha]^{23}_D$ +61.8 (*c* = 0.25, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.87 (s, 3H), 1.90 (s, 3H), 2.02 (s, 3H), 2.03 (s, 3H), 2.11 (s, 3H), 2.15 (s, 3H), 2.27 (dt, J = 13.0, 1.7 Hz, 1H), 2.53 (dd, J = 12.9, 4.6 Hz, 1H), 2.77 (d, J = 8.9 Hz, 1H), 3.29 (d, J = 4.4 Hz, 1H), 3.69–4.57 (m, 12H), 4.84–4.87 (m, 2H), 5.16 (d, J = 9.2 Hz, 1H), 5.02–5.34 (m, 6H), 5.39 (dt, J = 11.0, 5.4 Hz, 1H), 5.50 (d, J = 9.6 Hz, 1H), 5.88 (d, J = 8.2 Hz, 1H), 7.22–7.47 (m, 9H), 7.60 (d, 2H), 7.75 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 171.1, 170.8, 170.5, 170.3, 170.1, 170.0, 169.6, 169.0, 168.0, 155.8, 143.7, 143.6, 141.1, 135.0, 128.5, 128.4, 127.6, 127.0, 125.1, 125.0, 119.0, 99.2, 98.6, 94.8, 72.7, 69.2, 69.0, 68.8, 68.1, 67.5, 63.3, 62.7, 62.5, 60.1, 54.4, 53.2, 53.0, 49.1, 46.9, 36.8, 36.0, 22.9, 20.9, 20.8, 20.7, 20.6; IR (film) 3357, 3067, 2956, 2110, 1745, 1664, cm^{-1}. FAB HRMS *m/e* calcd for (M + Na) $C_{51}H_{59}N_5O_{21}Na$ 1100.3600, found 1100.3589.

Tetrasaccharide 42. Sialyated acceptor **41** (58 mg, 0.054 mmol) and thioglycoside **35** (22 mg, 0.027 mmol) were azeotroped with benzene (3 × 5 mL). NIS (15.2 mg, 0.068 mmol), 0.1 g of 4 Å mol sieves, and 2.0 mL of CH_2Cl_2 were then added. A freshly prepared solution of triflic acid (1% solution in CH_2Cl_2, 0.24 mL) was then added dropwise. After 5 min, the reaction was judged complete by TLC and quenched with triethylamine. Flash chromatography (3 → 3.5 → 4 → 4.5 → 5% MeOH in CH_2Cl_2) afforded 26 mg (53%) of **42** as a white film: $[\alpha]^{23}_D$ +20.8 (*c* = 1.25, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.02 (d, J = 6.7 Hz, 2H), 7.77 (d, J = 6.7 Hz, 2H), 7.60 (t, J = 6.8 Hz, 2H), 7.53 (t, J = 7.2 Hz, 1H), 7.04–7.44 (m, 11H), 5.84 (d, J = 8.3 Hz, 1H), 5.51 (dt, J = 10.7, 5.4 Hz, 1H), 5.16–5.38 (m, 10H), 5.06 (bs, 1H), 4.85 (bm, 1H), 4.77 (d, J = 7.9 Hz, 1H), 4.75 (bs, 1H), 4.61 (bd, J = 8.3 Hz, 2H), 3.75–4.48 (m, 22H), 3.65 (d, J = 10.5 Hz, 1H), 3.55 (dd, J = 9.7, 5.8 Hz, 1H), 3.48 (dd, J = 10.4, 3.4 Hz, 1H), 2.61 (bs, 1H), 2.56 (dd, J = 12.8, 4.6 Hz, 1H), 2.51 (dd, J = 13.9, 5.5 Hz, 1H), 2.12 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.87 (s, 3H), 1.86 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 171.0, 170.9, 170.7, 170.6, 170.4, 170.3, 170.2, 170.0, 169.9, 169.8, 168.0, 165.3, 163.0, 155.8, 143.8, 143.7, 141.2, 135.0, 133.4, 129.7, 129.1, 128.6, 128.5, 128.4, 128.3, 127.8, 127.1, 125.2, 120.0, 100.8, 99.0, 98.7, 95.1, 72.8, 72.7, 72.2, 71.2, 69.4, 69.2, 69.0, 68.9, 68.8, 68.0, 67.7, 67.6, 67.2, 67.0, 66.3, 62.5, 62.0, 58.3, 54.4, 53.4, 52.8, 49.3, 47.1, 38.0, 37.5, 29.7, 23.1, 23.0, 21.0, 20.8, 20.7, 20.6, 20.5; IR (film) 3366, 3065, 2959, 2111, 1744, 1687, 1533, 1369, 1225 cm^{-1}. FAB HRMS *m/e* calcd for (M + Na) $C_{85}H_{98}N_6O_{39}Na$ 1849.5767, found 1849.5766.

Hexasaccharide 45. A mixture of thioglycoside **43** (70.8 mg, 0.05 mmol) and 3-acceptor **44** (68.3 mg, 0.1 mmol) was azeotroped with toluene (3 × 5 mL). To the mixture was added powdered 4 Å mol sieves (0.2 g), and 3.0 mL of CH_2Cl_2, and the mixture was stirred 30 min at ambient temperature. The mixture was cooled to 0 °C, at which time NIS (26.7 mg, 0.12 mmol) and freshly prepared triflic acid solution (1% solution in CH_2Cl_2, 0.42 mL, 0.05 mmol) were added. The red mixture was stirred at 0 °C for 15 min and then diluted with EtOAc. The organic phase was washed with sat. $NaHCO_3$, 10% $Na_2S_2O_3$ and brine, dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography (1:1 → 2:1 EtOAc:CH_2Cl_2) to afford 80.1 mg (79%) of **45** as a colorless solid. $[\alpha]^{23}_D$ −26.4 (*c* 1.00, $CHCl_3$); ^1H

NMR ($CDCl_3$) δ 8.10 (d, J = 7.4 Hz, 2H), 7.79 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.0 Hz, 2H), 7.54 (t, J = 7.2 Hz, 1H), 7.43−7.24 (m, 12H), 5.86 (d, J = 8.5 Hz, 1H), 5.52−5.47 (m, 2H), 5.35−5.32 (m, 4H), 5.18−5.05 (m, 5H), 5.04−4.98 (m, 3H), 4.95−4.88 (m, 3H), 4.80 (d, J = 7.9 Hz, 1H), 4.72 (d, J = 3.3 Hz, 1H), 4.59−4.56 (m, 2H), 4.51 (dd, J = 11.7, 5.7 Hz, 1H), 4.43−4.37 (m, 2H), 4.33−4.23 (m, 2H), 4.21−4.07 (m, 6H), 4.03−3.84 (m, 5H), 3.80−3.73 (m, 4H), 3.44 (d, J = 10.3 Hz, 1H), 3.43 (d, J = 10.5 Hz, 1H), 3.21−3.13 (m, 1H), 2.83 (s, 1H), 2.21 (s, 3H), 2.18 (s, 3H), 2.16 (s, 3H), 2.14 (s, 3H), 2.12 (s, 3H), 2.11 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.99 (s, 6H), 1.27 (s, 3H), 1.14 (d, J = 5.6 Hz, 6H), 0.86 (s, 9H), 0.04 (s, 6H); ^{13}CNMR ($CDCl_3$) δ 171.37, 171.23, 171.10, 170.96, 170.91, 170.87, 170.85, 170.74, 170.54, 170.39, 170.17, 169.96, 169.92, 165.79, 156.31, 144.18, 141.69, 135.43, 134.09, 130.24, 129.51, 129.05, 129.01, 128.92, 128.84, 128.17, 127.50, 125.58, 125.54, 120.43, 102.39, 100.83, 100.69, 99.87, 96.62, 96.09, 78.11, 77.30, 74.25, 73.76, 73.52, 73.30, 72.96, 72.04, 71.81, 71.33, 71.26, 71.10, 71.03, 69.81, 69.38, 68.71, 68.61, 68.23, 68.10, 67.99, 67.95, 67.67, 67.29, 65.45, 64.36, 62.95, 62.20, 60.95, 58.84, 58.76, 54.87, 47.51, 26.25, 22.97, 21.47, 21.30, 21.26, 21.14, 21.08, 21.05, 20.99, 18.69, 16.28, 15.99, −4.98, −5.07; IR (neat): 2935, 2110, 1746 cm^{-1}. FAB HRMS *m/e* calcd for (M + Na) $C_{100}H_{127}N_5O_{45}SiNa$: 2168.7470; found 2168.7545.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **9**, **15**, **16**, **17**, **18**, **19**, **25**, **26**, **27**, **33**, **35**, **37**, **39**, **39b**, **40**, **41**, **42**, **45**; ^{1}H NMR spectra for compounds **11**, **12**, **14**; full chemical names for new compounds **25**, **27**, **33**, **39**, **40**, **41**, **42**, **45** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Chemistry

Probing cell-surface architecture through synthesis: An NMR-determined structural motif for tumor-associated mucins

DAVID H. LIVE*, LAWRENCE J. WILLIAMS†, SCOTT D. KUDUK†, JACOB B. SCHWARZ†, PETER W. GLUNZ†, XIAO-TAO CHEN‡, DALIBOR SAMES‡, R. AJAY KUMAR§, AND SAMUEL J. DANISHEFSKY†‡¶

*Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota Medical School, Minneapolis, MN 55455; †Laboratory for Bioorganic Chemistry, Sloan–Kettering Institute for Cancer Research, 1275 York Avenue, Box 106, New York, NY 10021; ‡Department of Chemistry, Columbia University, Havemeyer Hall, New York, NY 10027; and §Computational Biology Center, IBM Thomas J. Watson Research Center, Yorktown Heights, NY 10598

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ABSTRACT **Cell-surface mucin glycoproteins are altered with the onset of oncogenesis. Knowledge of mucin structure could be used in vaccine strategies that target tumor-associated mucin motifs. Thus far, however, mucins have resisted detailed molecular analysis. Reported herein is the solution conformation of a highly complex segment of the mucin CD43. The elongated secondary structure of the isolated mucin strand approaches the stability of motifs found in folded proteins. The features required for the mucin motif to emerge are also described. Immunocharacterization of related constructs strongly suggests that the observed epitopes represent distinguishing features of tumor cell-surface architecture.**

The exterior of most cells is dominated by glycolipids, proteoglycans, and glycoproteins, including mucin-like proteins. These display polyvalent α-*O*-linked carbohydrates on proximal serine and threonine residues (1–4). Altered expression of cell-surface mucin character is often characteristic of malignant cells (1, 3, 5). Accordingly, tumor-associated mucins are good targets for a vaccination strategy (3, 5–9). We have surmounted the synthetic challenges of constructing polypeptides bearing clustered glycodomains (7, 8), and one such construct is currently in human clinical trials. Given this access, we probed the effects of clustered glycosylation patterns on peptide conformation and recognition (9–18). We have conducted extensive NMR and restrained molecular dynamics calculations (19, 20) on fully synthetic clustered carbohydrate tumor antigens (**1–4**) corresponding to a fragment of CD43, a glycoprotein aberrantly expressed on the surface of acute myelogenous leukemia cells (21–24). Our findings demonstrate how clustered glycosylation induces the peptide backbone into an unprecedented rigid scaffold corresponding to a polypeptide secondary conformation, which is consistent with elongated mucin glycoprotein structure and function (1, 25–29). Remarkably, the glycosylation-induced structure approaches the stability of motifs found in globular proteins.

The CD43, or leukosialin, protein presented an ideal candidate from which to select a substructure for synthesis. In consequence of its possible role in inducing immunologic response, the system is relatively well characterized (22–24). The sequence STTAV is a glycosylation locus found in the amino terminus of the protein. We have accessed through chemical synthesis the clustered 2,6-STF trisaccharide **1** (7), which is present on CD43 when expressed on acute myelogenous leukemia cells (24). In addition, we have synthesized the TF disaccharide **2** and the monosaccharide Tn antigen **3**. Through our synthetic methods, we also gained access to the β-linked stereoisomer of the TF antigen **4** (Fig. 1) (8). As will be seen, the latter served as a critical control to assess the sensitivity of the glycosylation-induced structure to anomeric stereochemistry.

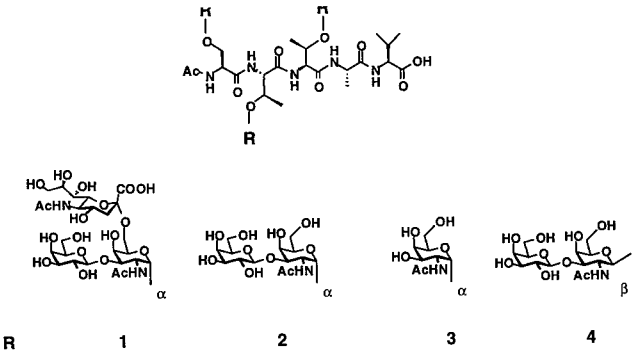

FIG. 1. Polypeptides **1–4** were prepared by total synthesis and represent clustered α- and β-*O*-linked glycodomains. The sequence STTAV is in the amino terminus of the protein CD43. The glycans, indicated as R, are known tumor antigens. The STF trisaccharide antigen of **1** is present on CD43 when expressed on acute myelogenous leukemia cells. The TF antigen of **2** and Tn antigen of **3** are expressed on certain malignant carcinomas, particularly of the prostate and colon. Constructs **1–3** contain the natural mucin α-*O*-GalNAc core. Construct **4** was prepared for comparison and has the unnatural β-linkage.

At the outset, we were mindful that, to date, mucin proteins have resisted detailed NMR analysis and that spectral resonances of highly glycosylated proteins lack the dispersion necessary to permit sequence assignment (16). The main difficulties arise from the presence of tandemly repeated peptide segments rich in serine and threonine. The first carbohydrate of *O*-linked mucins is α-*O*-GalNAc, and most of the serine and threonine residues are thought to be glycosylated (1, 4). Accordingly, it was of great interest to find that the amide region of the ^{1}H-NMR spectrum of constructs **1–3** revealed a dramatic alteration in the structure of the peptide backbone as a consequence of the α-linked carbohydrates (Fig. 2*a*). The structural change of the α-linked series further manifests itself in the significant increase in the lifetime of exchangeable peptide backbone amide protons (NH) relative to the free peptide. By contrast, the β-linked isomer **4** showed rather meager changes in amide chemical shifts relative to the α-isomer **2**. Furthermore, the exchange lifetimes of the NH of the GalNAc residues are very sensitive to anomeric stereochemistry. For example, where the β-linked GalNAc NH in

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Abbreviations: NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, Biology Department, Brookhaven National Laboratory, Upton, NY 11973 (PDB ID code 1sia).

¶To whom reprint requests should be addressed. e-mail: s-danishefsky@ski.mskcc.org.

compound **4** exchange in minutes, the corresponding NH of **1** persist for more than 12 hr. *These results demonstrate that sequential O-glycosylation by means of α-linked GalNAc induces transition to a highly stable structure*—a remarkable result in such a relatively small peptide.

To elucidate the conformation of the α-linked STF cluster in detail, extensive two-dimensional NMR homo- and heteronuclear NMR experiments, as well as a three-dimensional Total correlation spectroscopy (TOCSY)–nuclear Overhauser effect spectroscopy (NOESY) experiments were conducted at 600 and 800 MHz in H_2O and D_2O[‖]**. Whereas the presence of the identical pendant glycans present formidable challenges in resonance assignment, we were able to fully assign the amino acid residues and have identified the H1, H2, H3, NH, and methyl groups of each proximal GalNAc. The proton and proton–carbon heteronuclear multiple quantum coherence resonances for the peripheral galactose and sialic acid residues were found to be degenerate. Remarkably, we were able to correlate over 100 proton NOEs. These measurements reveal a strong interaction between the methyl protons of the GalNAc acetyl groups and the peptide for **1** (Fig. 2*b*). Similar interactions were observed for **2** and **3**. By contrast, no such interactions were observed in the "nonnatural" β-linked construct **4**. (For an in-depth analysis of the conformational symbiosis between β-*O*-linked Lewis X glycans and a segment of MAdCAM-1, see ref. 30. For earlier papers on this subject, see refs. 31–33.)

Fig. 3 depicts the solution structures of **1** calculated by restrained molecular dynamics in torsion angle space guided by data from multidimensional NMR (19, 20)[‖]**. The anomeric torsions are consistent with expected values based on the exoanomeric effect. Whereas the peptide backbone torsions do not match secondary structural motifs common in globular proteins, they fall in allowed regions of the Ramachandran map (see below). Comparison of the calculated structures revealed a remarkably well ordered core (with a rms deviation of only 1.17 ± 0.55 Å) consisting of the peptide backbone and the proximally linked glycans (Table 1). *Thus, compound* ***1*** *displays an unprecedented degree of order for a pentapeptide that approaches the structural stability of motifs found in folded proteins.*

The structure, as organized, displays two faces, one of which is primarily a carbohydrate surface, whereas the other presents a comparatively smaller peptide component. This structure is consistent within the larger mucin context, where the carbohydrate is directed to the exterior while the polypeptide is elongated to maximize accessibility of the glycodomain (1). The paucity of NOE interactions between the peripheral sugars and the core glycodomain suggests that distal glycan components play little role in determining the core mucin structure. Indeed, when **2** and **3** were examined in comparison with **1**, the NOE patterns corresponding to the core residues were virtually identical to those of the trisaccharide cluster. This homology of **1–3**, which does not extend to **4**, indicates the specific role of the α-linkage, but not the β-linkage, in inducing the secondary structure observed. The strong NOEs between the methyls of the GalNAc acetyl and the peptide indicate the GalNAc acetyls are probably necessary to maintain the observed structure, consistent with *N*-acetyl dependent conformation of monoglycosylated peptides (10, 13, 18). Thus, the core glycodomain, comprised of

Table 1. Statistical analysis of NMR and computed structural data for **1**

Distance restraints	Observed
Total	116
Intraresidue	
Pentapeptide	23
Glycans	10
Sequential ($\|k-j\| = 1$)	
Pentapeptide	28
Glycans	8
Medium range ($2 \leqq \|i-j\| \leqq 4$)	
Pentapeptide	1
Glycans	2
Pentapeptide–Glycans	
Self*	29
Other†	13
3-bond *J*-coupling restraints	
Pentapeptide	5
Glycans	6
Structure Statistics	**Value**
NOE violations	
Number >0.2 Å	2
Number >0.5 Å	0
Three-bond *J*-coupling violations	
Number >0.25 Hz	0
Deviations from ideal covalent geometry	
Bond length, Å	0.013 ± 0.005
Bond angle, Å	2.7 ± 0.4
Impropers, deg	1.3 ± 0.4
Pairwise rms deviation among 20 final structures, Å	
Peptapeptide backbone + (S1G1, T2G1, T3G1) rings	1.17 ± 0.55
Pentapeptide + (S1G1, T2G1, T3G1) heavy atoms	1.36 ± 0.60

*Between peptide residue and its attached glycans. These NOEs were limited to the proximally linked GalNac *N*-acetyl-methyl group.
†Between peptide residue and glycans on other peptide residues.

‖Compounds **1–4** were synthesized as described (see refs. 7 and 8). Samples of synthetic peptide and the synthetic glycopeptides were dissolved in D_2O or 90% H_2O/10% D_2O with 10 mM phosphate buffer for NMR analysis. The pH was adjusted in each case to ≈4.5. Sample concentrations were between 5 and 20 mM. NMR experiments were run at 18°C on Varian INOVA 600 and 800 spectrometers. 1D proton, two-dimensional heteronuclear multiple quantum coherence, heteronuclear multiple bond correlation, and double quantum correlation spectroscopy experiments were run in D_2O. One-dimensional proton, two-dimensional TOCSY (45-ms mixing period), NOESY (350-ms mixing period), and three-dimensional TOCSY–NOESY (45-ms and 500-ms mix times) were acquired in the H_2O/D_2O solvent by using the WATERGATE method for suppressing the solvent signal. The NOESY and TOCSY–NOESY experiments were used to determine NOE restraints for structure calculations, the one-dimensional experiment to determine the backbone couplings, and the other experiments for resonance assignments. Interproton distances were obtained from analysis of cross-peak intensities in NOESY spectra of **1** and were classified into three categories with corresponding bounds: strong (2.4 ± 0.6 Å), medium (2.9 ± 1.1 Å), and weak (3.4 ± 1.6 Å). Initial structural model of **1** was constructed in extended geometry (with sugars in chair form) by using INSIGHT II/BIOPOLYMER [Molecular Simulations (MSI), San Diego, CA]. Atom types and force field parameters were assigned corresponding to PARALLHDG.PRO (Version 4.02, M. Nilges) for the pentapeptide. For the glycans, ideal geometry values were taken from CHARMM19 (MSI) with bond, angle, and improper force constants set to match those in PARALLHDG.PRO. Nonbonded interactions were computed with the van der Waals term; the electrostatic term was turned off. The structure was minimized with the covalent energy terms to idealize the covalent geometry in the present force field. Peptide backbone and sugar linkage torsions were randomized to generate starting conformations. Structure refinement was carried out with distance and three-bond *J*-coupling restrained torsion angle dynamics in X-PLOR Version 98.0 (MSI) (see ref. 20) by using an optimized version (MSI) of the TAD protocol (see ref. 19), during which the conformation of each sugar ring was maintained rigid. The simulated annealing protocol consisted of 15 ps of dynamics computed with a time step of 15 fs at 50,000 K followed by cooling to 0 K in 15 ps and 2,000 steps of energy minimization. From a total of 100 computations, 20 final structures were chosen based on the criterion of least restraint violation. Color figures were prepared with INSIGHTII.

**Coordinates have been deposited with the Protein DataBank

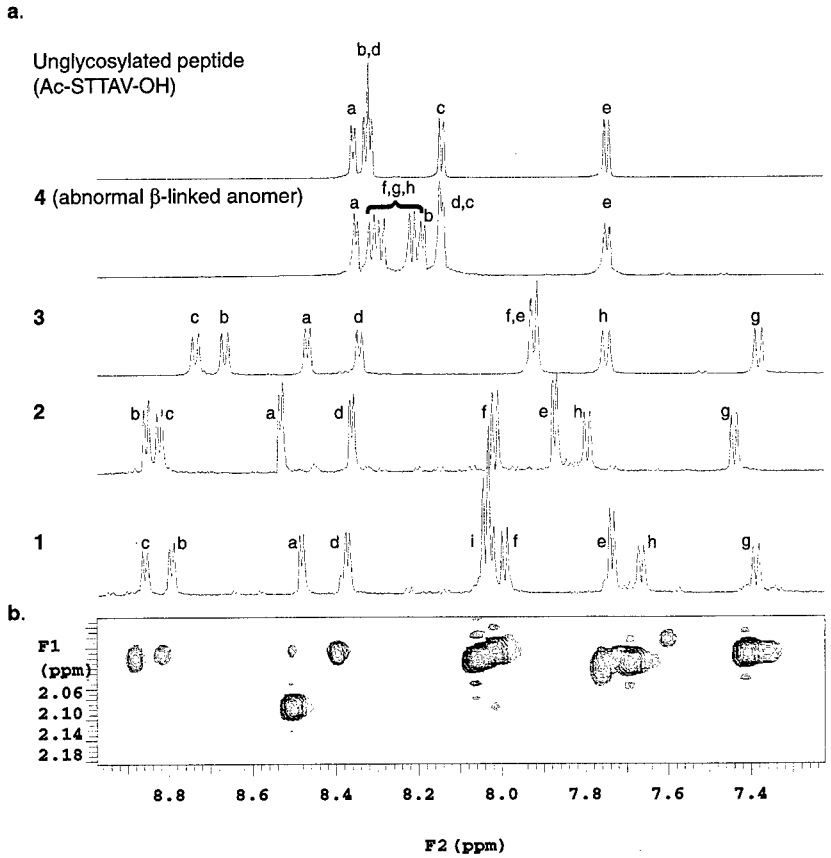


FIG. 2. One- and two-dimensional NMR analysis‖ demonstrates that sequential *O*-glycosylation of α-linked GalNAc induces transition to a highly stable structure. (*a*) The amide region of the ^{1}H-NMR for the peptide Ac-STTAV-OH, the β-linked **4**, and the α-linked series **3**, **2**, and **1**. The amide proton resonances of the peptide backbone, STTAV, are labeled a, b, c, d, and e, respectively. The GalNAc amide resonances on the STT segments of **1**–**4** are labeled f, g, and h, respectively, and the sialic acid amide signals are labeled i. Comparison of these spectra shows that NH patterns in abnormal (β-linked) anomer **4** differ little from the unglycosylated peptide in contrast to the spectra of α-linked clusters, which reveal dramatic dispersion of resonances. (*b*) Two-dimensional NOESY measurements reveal strong interactions between the methyl protons of the GalNAc acetyl groups and the peptide amide protons for **1**. Similar interactions were observed for α-linked **2** and **3**. No such interactions were observed in the β-linked **4**.

an amino acid and an α-*O*-GalNAc, dictates the organization of the mucin glycopeptide backbone into a scaffold on which the carbohydrate extensions are mounted, relatively unhindered in their conformational disposition, allowing the display of antennary glycans.

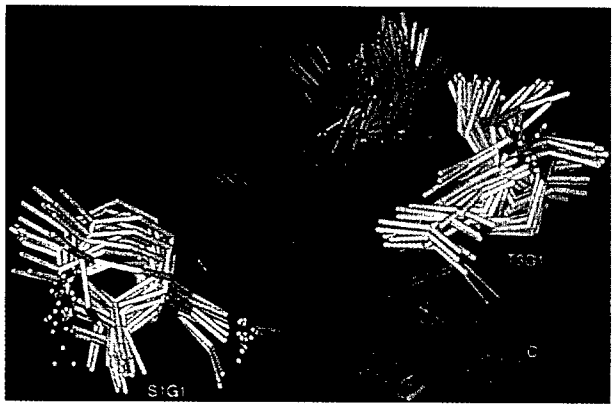
The stability of the core conformation is apparent from our data and the peptide backbone angles fall in allowed regions. However, the overall fold does not fit into one of the canonical classes of polypeptide secondary structure.** This organization is apparently because of conformational accommodations necessary to form a compact structure that also incorporates large branching sidegroups (starting with the proximal GalNAc), which have no counterpart in a nonglycosylated peptide of comparable size. Whereas the molecular details of the motif are novel, the elongated peptide dimensions are consistent with dimensions derived from electron micrographs of cell-surface mucin proteins (26–28). Indeed, the persistence of the backbone fold in the series of analogues we have examined demonstrates that the elongated secondary structure is energetically stable and suggests that *this may be a common motif in the nonglobular structure of mucin glycoproteins*.

We have reported immunological characterization of constructs related to **1**, **2**, and **3** and have shown that they elicit robust antibody responses that crossreact with tumor cells displaying the corresponding antigen (8). Hence, Fig. 3 also represents the epitope recognized by antibodies stimulated by our potential vaccines and the probable epitope of other vaccine candidates and related structures (6, 21). Tumor-associated mucins have the same GalNAc core as normal mucins, and the structure we observed is independent of antennary glycans. Thus, normal mucins should present the same scaffold as tumor-related mucins, except that they are more highly glycosylated, effectively concealing the carbohydrate scaffold and the proximal peptides (1, 5).

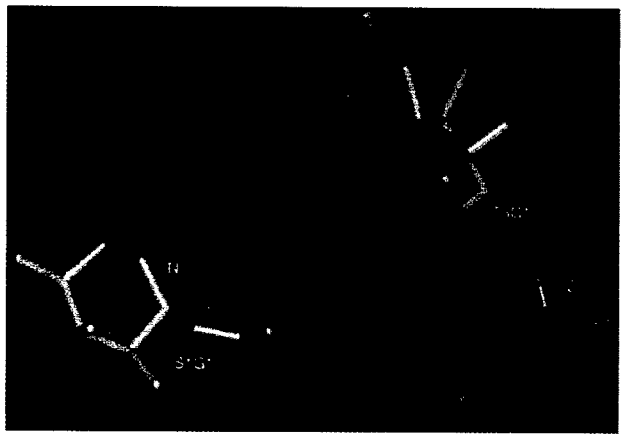
In combination with other conformational studies of *O*-glycosylated peptides, our findings suggest a progression from flexible peptide to the stable elongated structure of mucin proteins. Peptide flexibility is reduced on monoglycosylation with GalNAc (12, 18) and further restricted on addition of a second GalNAc (3, 9). These low degrees of glycosylation result in modest β-turn formation usually in the vicinity of the glycodomain and appear to be sequence sensitive. On formation of the clustered triad, the structure converges to a stable elongated motif. Incomplete biosynthetic elaboration of the glycan core, like that associated with certain carcinomas (1, 3, 5, 24), results in less ordered cell-surface architectures. Subsequent glycosylation in the normal course, leading to fully mature mucins, yields a stable elongated protein that polyvalently displays the glycans necessary for functional recognition (1, 4, 25–29).

In summary, sequential *O*-glycosylation of α-linked GalNAc induces a transition to an unprecedented secondary structure that approaches the stability of motifs found in folded proteins. We have shown that the α-linkage and sequential placement of GalNAc are required for this mucin motif to emerge. It is likely that the acetyl group on the GalNAc residue is also necessary to support structural coherence. Furthermore, installation of the initial α-*O*-

a



b



c

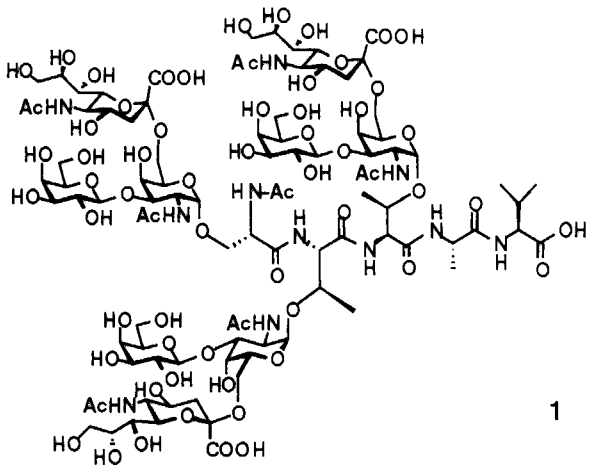


FIG. 3. Structure of the mucin glycopeptide 1[||]. (*a*) Superimposed view of the 20 final structures. The superposition was performed on the backbone atoms of the peptides and the ring atoms of the displayed sugars. The relatively unrestrained and unstructured distally linked glycans are omitted for clarity. The amino and acid termini are labeled N and C, respectively. The α-*O*-GalNAc residues are labeled S1G1, T2G1, and T3G1 for the STT segment. Carbon, nitrogen, and oxygen atoms are colored green, blue, and red, respectively, on the pentapeptide. (*b*) View of the structure that has lowest rms deviation to the average of the 20 structures shown in *a*. (*c*) STF cluster **1**.

GalNAc residue in a cluster domain creates a stable scaffold that can accept, without intrinsic change, increased glycosylation. We note that a variety of carbohydrate structures can be accommodated in this way so that the same protein backbone can display a variety of glycans, the nature of which reflect the physiological state of the cell.

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